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(57) Abstract

A method for producing chimeric influenza virus comprising transfection of a host cell with a recombinant ribonucleoprotein by electroporation and infection with a parental strain of influenza is described. Transfection of a host cell with a recombinant ribonucleoprotein by by electroporation for recombinant gene expression is also described. Chimeric influenza viruses comprising heterologous influenza M protein coding sequence, or comprising heterologous influenza NP coding sequence, are also described. The heterologous influenza coding sequence may contain at least one substitution of a native residue with a non-native residue.

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CHIMERIC INFLUENZA VIRUS AND ELECTROPORATION METHOD

The present invention relates to recombinant negative strand virus RNA templates which may be used to express heterologous gene products in appropriate host cell systems and/or to construct recombinant viruses that express, package, and/or present the heterologous gene product. The expression products and chimeric viruses may advantageously be used in vaccine formulations.

The invention is demonstrated by way of examples in which recombinant influenza virus RNA templates containing a heterologous gene coding sequences in the negative-polarity were constructed. These recombinant templates, when combined with purified viral RNA-directed RNA polymerase, were infectious, replicated in appropriate host cells, and expressed the heterologous gene product at high levels. In addition, the heterologous gene was expressed and packaged by the resulting recombinant influenza viruses.

1. BACKGROUND

A number of DNA viruses have been genetically engineered to direct the expression of heterologous proteins in host cell systems (e.g., vaccinia virus, baculovirus, etc.). Recently, similar advances have been made with positive-strand RNA viruses (e.g., poliovirus). The expression products of these constructs, i.e., the heterologous gene product or the chimeric virus which expresses the heterologous gene product, are thought to be potentially useful in vaccine formulations (either subunit or whole virus vaccines). One drawback to the use of viruses such as vaccinia for constructing recombinant or chimeric viruses for use in vaccines is the lack of variation in its major epitopes. This lack of variability in the viral strains places strict limitations on the repeated use of chimeric vaccinia, in that multiple vaccinations will generate host-resistance to the strain so that the inoculated virus cannot infect the host. Inoculation of a resistant individual with chimeric vaccinia will, therefore, not induce immune stimulation.

By contrast, influenza virus, a negative-strand RNA virus, demonstrates a wide variability of its major epitopes. Indeed, thousands of variants of influenza have been identified; each strain evolving by antigenic drift. The negative-strand viruses such as

influenza would be attractive candidates for constructing chimeric viruses for use in vaccines because its genetic variability allows for the construction of a vast repertoire of vaccine formulations which will stimulate immunity without risk of developing a tolerance. However, achieving this goal has been precluded by the fact that, to date, it has not been possible to construct recombinant or chimeric negative-strand RNA particles that are infectious.

a. THE INFLUENZA VIRUS

Virus families containing enveloped single-stranded RNA of the negative-sense genome are classified into groups having non-segmented genomes (Paramyxoviridae, Rhabdoviridae) or those having segmented genomes (Orthomyxoviridae, Bunyaviridae and Arenaviridae). The Orthomyxoviridae family, described in detail below, and used in the examples herein, contains only the viruses of influenza, types A, B and C.

The influenza virions consist of an internal ribonucleoprotein core (a helical nucleocapsid) containing the single-stranded RNA genome, and an outer lipoprotein envelope lined inside by a matrix protein (M). The segmented genome of influenza A consists of eight molecules (seven for influenza C) of linear, negative polarity, single-stranded RNAs which encode ten polypeptides, including: the RNA-directed RNA polymerase proteins (PB2, PB1 and PA) and nucleoprotein (NP) which form the nucleocapsid; the matrix proteins (M1, M2); two surface glycoproteins which project from the lipoprotein envelope: hemagglutinin (HA) and neuraminidase (NA); and nonstructural proteins whose function is unknown (NS1 and NS2). Transcription and replication of the genome takes place in the nucleus and assembly occurs via budding on the plasma membrane. The viruses can reassort genes during mixed infections.

Influenza virus adsorbs via HA to sialyloligosaccharides in cell membrane glycoproteins and glycolipids. Following endocytosis of the virion, a conformational change in the HA molecule occurs within the cellular endosome which facilitates membrane fusion, thus triggering uncoating. The nucleocapsid migrates to the nucleus where viral mRNA is transcribed as the essential initial event in infection. Viral mRNA is transcribed by a unique mechanism in which viral endonuclease cleaves the capped 5'-terminus from cellular heterologous mRNAs which then serve as primers for transcription of viral RNA templates by the viral transcriptase. Transcripts terminate at sites 15 to 22 bases from the ends of their templates, where oligo(U) sequences act

as signals for the template-independent addition of poly(A) tracts. Of the eight viral mRNA molecules so produced, six are monocistronic messages that are translated directly into the proteins representing HA, NA, NP and the viral polymerase proteins, PB2, PB1 and PA. The other two transcripts undergo splicing, each yielding two mRNAs which are translated in different reading frames to produce M1, M2, NS1 and NS2. In other words, the eight viral mRNAs code for ten proteins: eight structural and two nonstructural. A summary of the genes of the influenza virus and their protein products is shown in Table I below.

TABLE I
Influenza Virus Genome RNA Segments and Coding Assignments[a]

Segment	Length[b] (Nucleo-tides)	Encoded Poly-peptide[c]	Length[d] (Amino Acids)	Molecules Per Virion	Comments
1	2341	PB2	759	30-60	RNA transcriptase component; host cell RNA cap binding
2	2341	PB1	757	30-60	RNA transcriptase component; initiation of transcription; endonuclease activity?
3	2233	PA	716	30-60	RNA transcriptase component; elongation of mRNA chains?
4	1778	HA	566	500	Hemagglutinin; trimer; envelope glycoprotein; mediates attachment to cells
5	1565	NP	498	1000	Nucleoprotein; associated with RNA; structural component of RNA transcriptase

6	1413	NA	454	100	Neuraminidase; tetramer; envelope glycoprotein
7	1027	M_1	252	3000	Matrix protein; lines inside of envelope
		M_2	96		Structural protein in plasma membrane; spliced mRNA
		?	?9		Unidentified protein
8	890	NS_1	230		Nonstructural protein; function unknown
		NS_2	121		Nonstructural protein; function unknown; spliced mRNA

[a] Adapted from R.A. Lamb and P.W. Choppin (1983), Reproduced from the Annual Review of Biochemistry, Volume 52, 467-506.

[b] For A/PR/8/34 strain.

[c] Determined by biochemical and genetic approaches.

[d] Determined by nucleotide sequence analysis and protein sequencing.

Following transcription, virus genome replication is the second essential event in infection by negative-strand RNA viruses. As with other negative-strand RNA viruses, virus genome replication in influenza is mediated by virus-specified proteins. It is hypothesized that most or all of the viral proteins that transcribe influenza virus mRNA segments also carry out their replication. All viral RNA segments have common 3′ and 5′ termini, presumably to enable the RNA-synthesizing apparatus to recognize each segment with equal efficiency. The mechanism that regulates the alternative uses (i.e., transcription or replication) of the same complement of proteins (PB2, PB1, PA and NP) has not been clearly identified but appears to involve the abundance of free forms of one or more of the nucleocapsid proteins, in particular, the NP. The nucleus appears to be the site of virus RNA replication, just as it is the site for transcription.

The first products of replicative RNA synthesis are complementary copies (i.e., plus-polarity) of all influenza virus genome RNA segments (cRNA). These plus-stranded copies (anti-genomes) differ from the plus-strand mRNA transcripts in the structure of their termini. Unlike the mRNA transcripts, the anti-genomic cRNAs are not capped and methylated at the 5′ termini, and are not truncated and polyadenylated at the 3′ termini. The cRNAs are coterminal with their negative strand templates and contain all the genetic information in each genomic RNA segment in the complementary form. The cRNAs serve as templates for the synthesis of genomic negative-strand vRNAs.

The influenza virus negative strand genomes (vRNAs) and antigenomes (cRNAs) are always encapsidated by nucleocapsid proteins; the only unencapsidated RNA species are virus mRNAs. In contrast to the other enveloped RNA viruses, nucleocapsid assembly appears to take place in the nucleus rather than in the cytoplasm. The virus matures by budding from the apical surface of the cell incorporating the M protein on the cytoplasmic side or inner surface of the budding envelope. The HA and NA become glycosylated and incorporated into the lipid envelope. In permissive cells, HA is eventually cleaved, but the two resulting chains remain united by disulfide bonds.

It is not known by what mechanism one copy of each of the eight genomic viral RNAs is selected for incorporation into each new virion. Defective interfering (DI) particles are often produced, especially following infection at high multiplicity.

b. RNA DIRECTED RNA POLYMERASE

The RNA-directed RNA polymerases of animal viruses have been extensively studied with regard to many aspects of protein structure and reaction conditions. However, the elements of the template RNA which promote optimal expression by the polymerase could only be studied by inference using existing viral RNA sequences. This promoter analysis is of interest since it is unknown how a viral polymerase recognizes specific viral RNAs from among the many host-encoded RNAs found in an infected cell.

Animal viruses containing plus-sense genome RNA can be replicated when plasmid-derived RNA is introduced into cells by transfection (for example, Racaniello et al. 1981, Science 214: 916-919; Levis, et al., 1986, Cell 44: 137-145). In the case of poliovirus, the purified polymerase will replicate a genome RNA in in vitro reactions and when this preparation is transfected into cells it is infectious (Kaplan, et al., 1985, Proc. Natl. Acad. Sci. USA 82: 8424-8428). However, the template elements which serve as transcription promoter for the poliovirus-encoded polymerase are unknown since even RNA homopolymers can be copied (Ward, et al., 1988, J. Virol. 62: 558-562). SP6 transcripts have also been used to produce model defective interfering (DI) RNAs for the Sindbis viral genome. When the RNA is introduced into infected cells, it is replicated and packaged. The RNA sequences which were responsible for both recognition by the Sindbis viral polymerase and packaging of the genome into virus particles were shown to be within 162 nucleotides (nt) of the 5′ terminus and 19 nt of the 3′ terminus of the genome (Levis, et al., 1986, Cell 44: 137-145). In the case of brome mosaic virus (BMV), a positive strand RNA plant virus, SP6 transcripts have been used to identify the promoter as a 134 nt tRNA-like 3′ terminus (Dreher, and Hall, 1988, J. Mol. Biol. 201: 31-40). Polymerase recognition and synthesis were shown to be dependent on both sequence and secondary structural features (Dreher, et al., 1984, Nature 311: 171-175).

The negative-sense RNA viruses have been refractory to study of the sequence requirements of the replicase. The purified polymerase of vesicular stomatitis virus is only active in transcription when virus-derived ribonucleoprotein complexes (RNPs) are included as template (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 126: 40-49); Emerson and Yu, 1975, J. Virol. 15: 1348-1356; Naito, and Ishihama, 1976, J. Biol. Chem. 251: 4307-4314). RNPs have been reconstituted from naked RNA of VSV DI particles using infected cell extracts as protein source. These RNPs were then replicated when added back to infected cells (Mirakhur, and Peluso, 1988, Proc. Natl. Acad. Sci. USA 85: 7511-7515). With regard to influenza viruses, it was recently reported that naked RNA purified from virus was used to reconstitute RNPs. The viral nucleocapsid and polymerase proteins were gel-purified and renatured on the viral RNA using thioredoxin (Szewczyk, et al., 1988, Proc. Natl. Acad. Sci. USA, 85: 7907-7911). However, these authors did not show that the activity of the preparation was specific for influenza viral RNA, nor did they analyze the signals which promote transcription.

During the course of influenza virus infection the polymerase catalyzes three distinct transcription activities. These include the synthesis of (a) subgenomic mRNA, which contains a 5′ cap and a 3′ poly-A tail; (b) a full length plus-strand or anti-genome (cRNA) copied from the genome RNA; and (c) genomic vRNA synthesized from the full length cRNA (reviewed in Ishihama and Nagata, 1988, CRC Crit. Rev. Biochem. 23: 27-76; and Krug, Transcription and replication of influenza viruses. In: Genetics of influenza viruses, Ed., Palese, P. and Kingsbury, D.W. New York, Springer-Verlag, 1983, p. 70-98). Viral proteins PB2, PB1 and PA are thought to catalyze all influenza virus-specific RNA synthesis when in the presence of excess nucleocapsid protein (NP; see above reviews). These polymerase functions have been studied using RNP cores derived from detergent-disrupted virus, and RNPs from the nuclear extracts of infected cells. Transcription from the RNPs derived from disrupted virus occurs when primed with either dinucleotide adenylyl-(3′-5′)-guanosine (ApG) or capped mRNAs. The plus sense mRNA products have terminated synthesis 17-20 nucleotides upstream of the 5′ terminus of the RNA template and have been processed by the addition of poly A tails. These products cannot serve as template for the viral-sense genome since they lack terminal sequences (Hay, et al., 1977, Virology 83: 337-

355). RNPs derived from nuclear extracts of infected cells also synthesize polyadenylated mRNA in the presence of capped RNA primers. However, if ApG is used under these conditions, both RNAs, polyadenylated and full length cRNA, can be obtained (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286; Takeuchi, et al., 1987, J. Biochem. 101: 837-845). Recently it was shown that replicative synthesis of cRNA could occur in the absence of exogenous primer if the nuclear extract was harvested at certain times post infection. In these same preparations the synthesis of negative-sense vRNA from a cRNA template was also observed (Shapiro and Krug, 1988, J. Virol. 62: 2285-2290). The synthesis of full length cRNA was shown to be dependent upon the presence of nucleocapsid protein (NP) which was free in solution (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286; Shapiro and Krug, 1988, J. Virol. 62: 2285-2290). These findings led to the suggestion that the regulatory control between mRNA and cRNA synthesis by the RNP complex is based on the requirement for there being an excess of soluble NP (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286).

Another line of investigation has focused on the preparation of polymerase-RNA complexes derived from RNPs from detergent-disrupted virus. When the RNP complex is centrifuged through a CsCl-glycerol gradient, the RNA can be found associated with the three polymerase (P) proteins at the bottom of the gradient. Near the top of the gradient, free NP protein can be found (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Kato, et al., 1985, Virus Research 3, 115-127). The purified polymerase-RNA complex (bottom of gradient), is active in initiating ApG-primed synthesis of RNA, but fails to elongate to more than 12-19 nucleotides. When fractions from the top of the gradient containing the NP protein are added back to the polymerase-RNA complex, elongation can ensue (Honda, et al., 1987, J. Biochem. 102: 41-49). These data suggest that the NP protein is needed for elongation, but that initiation can occur in the absence of NP.

It has been shown that the genomic RNA of influenza viruses is in a circular conformation via base-pairing of the termini to form a panhandle of 15 to 16 nt (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). Since the viral polymerase was found bound to the panhandle, this led to the suggestion that a panhandle structure was required for

recognition by the viral polymerase (Honda, et al., 1988, J. Biochem. 104: 1021-1026). Therefore, it was hypothesized in these two reports that the promoter for the viral RNA polymerase was the double stranded RNA in panhandle conformation.

2. SUMMARY OF THE INVENTION

Recombinant negative-strand viral RNA templates are described which may be used with purified RNA-directed RNA polymerase complex to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. The RNA templates are prepared by transcription of appropriate DNA sequences with a DNA-directed RNA polymerase. The resulting RNA templates are of the negative-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template.

As demonstrated by the examples described herein, recombinant negative-sense influenza RNA templates may be mixed with purified viral polymerase proteins and nucleoprotein (i.e., the purified viral polymerase complex) to form infectious recombinant RNPs. These can be used to express heterologous gene products in host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with recombinant RNPs and virus. Alternatively, the recombinant RNA templates or recombinant RNPs may be used to transfect transformed cell lines that express the RNA dependent RNA-polymerase and allow for complementation. Additionally, a non-virus dependent replication system for influenza virus is also described. Vaccinia vectors expressing influenza virus polypeptides were used as the source of proteins which were able to replicate and transcribe synthetically derived RNPs. The minimum subset of influenza virus protein needed for specific replication and expression of the viral RNP was found to be the three polymerase proteins (PB2, PB1 and PA) and the nucleoprotein (NP). This suggests that the nonstructural proteins, NS1 and NS2, are not absolutely required for the replication and express of viral RNP.

The expression products and/or chimeric virions obtained may advantageously be utilized in vaccine formulations. The use of recombinant influenza for this purpose is especially attractive since influenza demonstrates tremendous strain variability allowing for the construction of a vast repertoire of vaccine formulations. The ability to select from thousands of influenza variants for constructing chimeric viruses obviates

the problem of host resistance encountered when using other viruses such as vaccinia. In addition, since influenza stimulates a vigorous secretory and cytotoxic T cell response, the presentation of foreign epitopes in the influenza virus background may also provide for the induction of secretory immunity and cell-mediated immunity.

a. DEFINITIONS

As used herein, the following terms will have the meanings indicated:

cRNA	=	anti-genomic RNA
HA	=	hemagglutinin (envelope glycoprotein)
M	=	matrix protein (lines inside of envelope)
MDCK	=	Madin Darby canine kidney cells
MDBK	=	Madin Darby bovine kidney cells
moi	=	multiplicity of infection
NA	=	neuraminidase (envelope glycoprotein)
NP	=	nucleoprotein (associated with RNA and required for polymerase activity)
NS	=	nonstructural protein (function unknown)
nt	=	nucleotide
PA, PB1, PB2	=	RNA-directed RNA polymerase components
RNP	=	ribonucleoprotein (RNA, PB2, PB1, PA and NP)
rRNP	=	recombinant RNP
vRNA	=	genomic virus RNA
viral polymerase complex	=	PA, PB1, PB2 and NP
WSN	=	influenza A/WSN/33 virus
WSN-HK virus:		reassortment virus containing seven genes from WSN virus and the NA gene from influenza A/HK/8/68 virus
PR8	=	influenza A/PR/8/34 virus

3. DESCRIPTION OF THE FIGURES

Figure 1. Purification of the polymerase preparation. RNP cores were purified from whole virus and then subjected to CsCl-glycerol gradient centrifugation. The polymerase was purified from fractions with 1.5 to 2.0 M CsCl. Samples were then analyzed by polyacrylamide gel electrophoresis on a 7-14% linear gradient gel in the presence of 0.1% sodium dodecylsulfate followed by staining with silver. Protein samples contained 1.4 μg whole virus (lane 1), 0.3 μg whole virus (lane 2), 5 μl of RNP cores (lane 3) and 25 μl RNA polymerase (lane 4). Known assignments of the proteins are indicated at the left.

Figure 2. Plasmid constructs used to prepare RNA templates. The plasmid design is depicted with the solid box representing pUC-19 sequences, the hatched box

represents the truncated promoter specifically recognized by bacteriophage T7 RNA polymerase, the solid line represents the DNA which is transcribed from plasmids which have been digested with MboII. The white box represents sequences encoding the recognition sites for MboII, EcoRI and PstI, in that order. Sites of cleavage by restriction endonucleases are indicated. Beneath the diagram, the entire sequences of RNAs which result from synthesis by T7 RNA polymerase from MboII-digested plasmid are given. The V-wt RNA has the identical 5′ and 3′ termini as found in RNA segment 8 of influenza A viruses, separated by 16 "spacer" nucleotides. The RNA, M-wt, represents the exact opposite stand, or "message-sense", of V-wt. Restriction endonuclease sites for DraI, EcoRI, PstI and SmaI are indicated. T7 transcripts of plasmids cleaved by these enzymes result in, respectively, 32, 58, 66 and 91 nucleotide long RNAs. The sequences of V-d5′ RNA are indicated. The plasmid design is essentially the same as that used for the V-wt RNA except for the minor changes in the "spacer" sequence. The point mutants of V-d5′ RNAs which were studied are indicated in Table I.

Figure 3. Analysis of products of influenza viral polymerase. FIG. 3A: Polymerase reaction mixtures containing 0.4 mM ApG (lane 2) or no primer (lane 3) were electrophoresed on 8% polyacrylamide gels containing 7.7 M urea. FIG. 3B: The nascent RNA is resistant to single-stranded specific nuclease S1. Following the standard polymerase reaction, the solutions were diluted in nuclease S1 buffer (lane 1) and enzyme was added (lane 2). As control for S1 digestion conditions, radioactively labeled single-stranded V-wt RNA was treated with nuclease S1 (lane 3) or with buffer alone (lane 4). FIG. 3C: Ribonuclease T1 analysis of gel-purified reaction products. The reaction products of the viral polymerase using the V-wt RNA template was subjected to electrophoresis on an 8% polyacrylamide gel. The 53 nt band and the smaller transcript were excised and eluted from the gel matrix. These RNAs were digested with RNAse T1 and analyzed by electrophoresis on a 20% polyacrylamide gel containing 7.7 M urea. For comparison, T7 transcripts of M-wt and V-wt RNAs which had been synthesized in the presence of a α-^{32}P-UTP were also analyzed with RNAse T1. The predicted radiolabeled oliognucleotides of the control RNAs are indicated. Lane 1, 53 nucleotide full length (FL) product; lane 2, 40-45 nucleotide

smaller (Sm) RNA product; lane 3, M-wt RNA labeled by incorporation of ^{32}P-UMP; and lane 4, V-wt RNA labeled as in lane 3.

Figure 4. Optimal reaction conditions for the viral polymerase. FIG. 4A: Reactions with V-wt template were assembled on ice and then incubated at the indicated temperatures for 90 minutes. FIG. 4B: Reactions with the V-wt template were prepared in parallel with the indicated NaCl or KCl concentrations and were incubated at 30°C for 90 minutes. FIG. 4C: A single reaction with the V-wt template was incubated at 30°C, and at the indicated times, samples were removed and immediately processed by phenol-chloroform extraction. All gels contained 8% polyacrylamide with 7.7 M urea.

Figure 5. Template specificity of the viral polymerase. FIG. 5A: The viral polymerase reaction requires 3′ terminal promoter sequences. Different template RNAs were used in reactions under standard conditions. Lane 1, the V-Pst RNA, which is identical to V-wt except it has a 13 nt extension at the 3′ end; lane 2, V-Sma RNA, which has a 38 nt extension at the 3′ end; lane 3, V-wt RNA; lane 4, a DNA polynucleotide with identical sequence as the V-wt RNA; lane 5, and 80 nt RNA generated by bacteriophage T3 RNA polymerase transcription of a pIBI-31 plasmid digested with HindIII. The autoradiograph was overexposed in order to emphasize the absence of specific reaction products when these other templates were used. FIG. 5B: 10 ng of each template RNA were incubated with the viral polymerase and the products were then subjected to electrophoresis on 8% polyacrylamide gels containing 7.7 M urea. Lane 1, V-wt RNA; lane 2, V-Dra RNA; lane 3, V-Eco RNA; lane 4, M-wt RNA are shown; and lane 5, a 53nt marker oligonucleotide. For the exact sequence differences refer to FIG. 2 and Section 6.1 et seq.

Figure 6. The RNA promoter does not require a terminal panhandle. Polymerase reaction using two template RNAs. Each reaction contained 5 ng of V-wt RNA. As a second template the reactions contained 0 ng (lane 1), 0.6 ng (lane 2), and 3.0 ng (lane 3) of V-d5′ RNA. The resulting molar ratios are as indicated in the figure. The reaction products were analyzed on an 8% polyacrylamide gel in the presence of 7.7 M urea. Following densitometry analysis of autoradiographs, the relative intensity of each peak was corrected for the amount of radioactive UMP which is incorporated in each product.

Figure 7. Specificity of promoter sequences. RNAs which lacked the 5′ terminus and contained point mutations (Table II) were compared with V-d5′ RNA in standard polymerase reactions. The right panel is from a separate reaction set. Quantitative comparisons is outlined in Table II.

Figure 8. High concentration polymerase preparations are active in cap-endonuclease primed and in primerless RNA synthesis reactions. FIG. 8A: Primer specificity of the high concentration enzyme. Radioactively synthesized 30 nt template is in lane 1. Reactions using 20 ng of V-d5′ RNA and 5 μl of viral polymerase contained as primer: no primer (lane 2); 100 ng BMV RNA (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 6: 40-49) containing a cap 0 structure (lane 3); 100 ng rabbit globin mRNA, containing a cap 1 structure, (lane 4); and 0.4 mM ApG (lane 5). A lighter exposure of lane 5 is shown as lane 6. FIG. 8B: Nuclease S1 analysis of gel-purified RNAs. Products from reactions using as primer ApG (lanes 1 and 2); no primer (lanes 3 and 4); or globin mRNA (lanes 5 and 6) were electrophoresed in the absence of urea and the appropriate gel piece was excised and the RNA was eluted. This RNA was then digested with nuclease S1 (lanes 2, 4, and 6) and the products were denatured and analyzed on an 8% polyacrylamide gel containing 7.7 M urea.

Figure 9. Genomic length RNA synthesis from reconstituted RNPs. Reaction products using 10 μl of polymerase and as template 890 nt RNA identical to the sequence of segment 8 of virus A/WSN/33 and RNA extracted from A/PR/8/34 virus were analyzed on a 4% polyacrylamide gel containing 7.7 M urea. In lane 1, the 890 nt template synthesized radioactively by T7 RNA polymerase is shown. The 890 nt plasmid-derived RNA was used as template in lanes 2, 3, 8 and 9. RNA extracted from virus was used as template in lanes 4, 5, 10 and 11. No template was used in lanes 6 and 7. No primer was used in lanes 2 to 5, and ApG was used as primer in lanes 6 to 11. Reaction products were treated with nuclease S1 in lanes 3, 5, 7, 9 and 11.

Figure 10. Diagrammatic representation of a PCR-directed mutagenesis method which can be used to replace viral coding sequences within viral gene segments.

Figure 11.(A). Diagrammatic representation of relevant portions of pIVCAT1. The various domains are labeled and are, from left to right; a truncated T7 promoter; the 5′ nontranslated end of influenza A/PR/8/34 virus segment 8 (22 nucleotides); 8

nucleotides of linker sequence; the entire CAT gene coding region (660 nucleotides) the entire 3′ nontranslated end of influenza A/PR/8/34 virus segment 8 (26 nucleotides); and linker sequence containing the HgaI restriction enzyme site.. Relevant restriction enzyme sites and start and stop sites for the CAT gene are indicated. (B) The 716 base RNA product obtained following HgaI digestion and transcription of pIVACAT1 by T7 RNA polymerase. Influenza viral sequences are indicated by bold letters, CAT gene sequences by plain letters, and linker sequences by italics. The triplets -- in antisense orientation -- representing the initiation and termination codons of the CAT gene are indicated by arrow and underline, respectively.

Figure 12. RNA products of T7 polymerase transcription and in vitro influenza virus polymerase transcription. Lanes 1-4: polyacrylamide gel analysis of radiolabeled T7 polymerase transcripts from pIVACAT1, and pHgaNS. Lanes 5 and 6: Polyacrylamide gel analysis of the radiolabeled products of in vitro transcription by purified influenza A polymerase protein using unlabeled 1VACAT1 RNA and HgaNS RNA templates. Lane 1: HgaNS RNA of 80 nt. Lanes 2-4: different preparations of IVACAT1 RNA. Lane 5: viral polymerase transcript of IVACAT1 RNA. Lane 6: viral polymerase transcript of HgaNS RNA.

Figure 13. Schematic of the RNP-transfection and passaging experiments.

Figure 14. CAT assays of cells RNP-transfected with IVACAT1 RNA. (A) Time course of RNP-transfection in 293 cells. Cells were transfected at -1 hour with the recombinant RNP and infected with virus at 0 hour. Cells were harvested at the indicated time points and assayed for CAT activity. (B) Requirements for RNP-transfection of 293 cells Parameters of the reaction mixtures were as indicated. (C) RNP-transfection of MDCK cells. MDCK cells were transfected with IVACAT1 RNA-polymerase at either -1 hour or +2 hours relative to virus infection. Cells were harvested and CAT activity assayed at the indicated times. Components/conditions of the reaction were as indicated. "Time" indicates the time point of harvesting the cells. T=0 marks the time of addition of helper virus. "RNA" represents the IVACAT1 RNA. "Pol" is the purified influenza A/PR/8/34 polymerase protein complex. "WSN" indicates the influenza A/WSN/33 helper virus. "Pre-Inc." indicates preincubation of RNA and polymerase in transcription buffer at 30°C for 30 min. "RNP transfection" indicates the time of RNP transfection relative to virus infection. "+/-" indicate

presence or absence of the particular component/feature. "C" indicates control assays using commercially available CAT enzyme (Boehringer-Mannheim).

Figure 15. CAT activity in MDCK cells infected with recombinant virus. Supernatant from RNP-transfected and helper virus-infected MDCK cells was used to infect fresh MDCK cells. The inoculum was removed 1 hour after infection, cells were harvested 11 hours later and CAT activity was assayed. Lane 1: extract of cells infected with helper virus only. Lane 2: extract of cells infected with 100 μl of supernatant from RNP-transfected and helper virus-infected MDCK cells. Lane 3: Supernatant (80 μl) of cells from lane 2. Lane 4: Same as lane 2 except that helper virus (MOI 4) was added to inoculum. In contrast to experiments shown in FIG. 4, the assays contained 20 μl of ^{14}C chloramphenicol.

FIG. 16. Diagram of relevant portions of the neuraminidase (NA) gene contained in plasmids used for transfection experiments. The pUC19 derived plasmid pT3NAv contains the influenza A/WSN/33 virus NA gene and a truncated promoter specifically recognized by bacteriophage T3 RNA polymerase. The T3 promoter used is truncated such that the initial transcribed nucleotide (an adenine) corresponds to the 5′ adenine of the WSN NA gene. At the 3′ end of the cDNA copy of the NA gene, a Ksp632I restriction enzyme site was inserted such that the cleavage site occurs directly after the 3′ end of the NA gene sequence. A 1409 nucleotide long transcript was obtained following Ksp632I digestion and transcription by T3 RNA polymerase of PT3NAv (as described in Section 8.1, infra). The 15 5′ terminal nucleotides, the 52 nucleotides corresponding to the region between the restriction endonuclease sites NcoI and PstI and the 12 3′ terminal nucleotides are shown. The transcript of pT3NAv mut 1 is identical to that of pT3NAv except for a single deletion, eleven nucleotides downstream from the 5′ end of the wild type RNA. The transcript of the pT3NAv mut 2 is identical to that of pT3NAv except for 5 mutations located in the central region (indicated by underline). These five mutations do not change the amino acid sequence in the open reading frame of the gene. The serine codon UCC at position 887-889 (plus sense RNA) was replaced with the serine codon AGU in the same frame. The numbering of nucleotides follows Hiti et al., 1982, J. Virol., 41: 730-734.

FIG. 17. Polyacrylamide gel electrophoresis of RNAs purified from rescued influenza viruses. RNA transcripts of pT3NAs (FIG. 16) of phenol-extracted RNA

derived from influenza A/WSN/33 virus was mixed with purified polymerase preparations following the protocol described in Section 6.1.1, infra. These reconstituted RNPs were then transfected into MDBK cells which had been infected one hour earlier with WSN-HK helper virus. The medium, containing 28 μg/ml plasminogen, was harvested after 16 hours and virus was amplified and plaqued on MDBK cells in the absence of protease. Virus obtained from plaques was then further amplified in MDBK cells and RNA was phenol-extracted from purified virus preparations as described in Sections 6.1 et seq. and 7.1 et seq. RNAs were separated on 2.8% polyacrylamide-0.075% bisacrylamide gels containing 7.7 M urea in TBE buffer and visualized by silverstaining as described in Section 6.1 et seq. Lanes 1 and 6: WSN-HK virus RNA. Lane 2: RNA of virus which was rescued from MDBK cells following RNP-transfection with pT3NAv derived NA RNA and infection with helper virus WSN-HK. Lane 3: NA RNA transcribed in vitro from pT3NAv. Lane 4: RNA of control WSN virus. Lane 5: RNA of virus which was rescued from MDBK cells following RNP-transfection with phenol-extracted WSN virus RNA and infection with helper virus WSN-HK.

FIG. 18. Sequence analysis of RNA obtained from rescued influenza virus containing five site-specific mutations. Following infection with the WSN-HK helper virus, MDBK cells were RNP-transfected with T3NAv mut 2 RNA which was obtained by transcription from pT3NAv mut 2. Following overnight incubation in the presence of 28 μg/ml plasminogen, medium was used for propagation and plaquing on MDBK cells in the absence of protease. Virus from plaques was then amplified and RNA was obtained following phenol-extraction of purified virus. Rescue of the mutant NA gene into virus particles was verified through direct RNA sequencing using 5′-TACGAGGAAATGTTCCTGTTA-3′ as primer (corresponding to position 800-819; Hiti et al., J. Virol., 41: 730-734) and reverse transcriptase (Yamashita et al., 1988, Virol. 163: 112-122). Sequences shown correspond to position 878-930 in the NA gene (Hiti et al., J. Virol. 41: 730-734). The arrows and the underlined nucleotides indicate the changes in the mutant RNA compared to the wild type RNA. Left: Control RNA obtained from influenza A/WSN/33 virus. Right: RNA of mutant virus rescued from MDBK cells which were RNP-transfected with T32NAv mut 2 RNA and infected with helper virus WSN-HK.

FIG. 19. CAT expression in vaccinia virus-infected/IVACAT-1 RNP transfected cells. Approximately 10^6 mouse C127 cells in 35 mm dishes were infected with mixtures of recombinant vaccinia viruses (Smith et al., 1986) at an M.O.I. of approximately 10 for each vector. After 1.5 hours, synthetic IVACAT-1 RNP was transfected into the virus-infected cells as described (Lutjyes et al., 1989). Cells were incubated overnight, harvested and assayed for CAT activity according to standard procedures (Gorman et al., 1982). The assays contained .05 uCl [^{14}C] chloramphenicol, 20 μl of 40 mM acetyl-CoA (Boehringer and 50 μl of cell extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were approximately 4 hours. The labels under the lane numbers indicate the treatment of cells. Lanes 1-control; 2-naked RNA transfection (no polymerase added), no helper virus infection; 3-RNP transfection, no helper virus; 4-RNP transfection, influenza virus as helper; Lanes 5-11-RNP transfection, vaccinia virus vectors as helper viruses express the indicated influenza virus proteins.

FIG. 20. Test of various cell lines. A) Cells were infected with vaccinia vectors expressing the PB2, PB1 and PA proteins (Lanes 1,3,5,7) or the PB2, PB1, PA and NP proteins (Lanes 2,4,6,8), transfected with IVACAT-1 RNP and examined for CAT activity as described. Lanes 1,2: Maden-Darby Canine Kidney (MDCK) cell; 3,4: Hela cells, 5,6: 293 cells (Graham et al., 1977 J. Gen. Virol., 36: 59-72); 7,8 L cells. B) Cell line 3 PNP-4 was used as host cell. Shown under each lane is the influenza viral proteins expressed in each sample. C) 293 cells were infected with the four required vaccinia and transfected with synthetic RNP made using IVA-CAT-I (lane 1) or IVA-CAT-2 (lane 2) RNA. After overnight incubation, cells were harvested and CAT assays were performed.

4. DETAILED DESCRIPTION

This invention relates to the construction and use of recombinant negative strand viral RNA templates which may be used with viral RNA-directed RNA polymerase to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. The RNA templates may be prepared by transcription of appropriate DNA sequences using a DNA-directed RNA polymerase such as bacteriophage T7, T3 or the Sp6 polymerase. Using influenza, for example, the DNA is constructed to encode the messagesense of the heterologous gene sequence

flanked upstream of the ATG by the complement of the viral polymerase binding site/promoter of influenza, i.e., the complement of the 3′- terminus of a genome segment of influenza. For rescue in virus particles, it may be preferred to flank the heterologous coding sequence with the complement of both the 3′-terminus and the 5′-terminus of a genome segment of influenza. After transcription with a DNA-directed RNA polymerase, the resulting RNA template will encode the negative polarity of the heterologous gene sequence and will contain the vRNA terminal sequences that enable the viral RNA-directed RNA polymerase to recognize the template.

The recombinant negative sense RNA templates may be mixed with purified viral polymerase complex comprising viral RNA-directed RNA polymerase proteins (the P proteins) and nucleoprotein (NP) which may be isolated from RNP cores prepared from whole virus to form "recombinant RNPs" (rRNPs). These rRNPs are infectious and may be used to express the heterologous gene product in appropriate host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with the rRNPs and virus. Alternatively, the recombinant RNA templates may be used to transfect transformed cell lines that express the RNA-directed RNA polymerase proteins allowing for complementation.

The invention is demonstrated by way of working examples in which RNA transcripts of cloned DNA containing the coding region -- in negative sense orientation -- of the chloramphenicol acetyltransferase (CAT) gene flanked by the 22 5′ terminal and the 26 3′ terminal nucleotides of the influenza A/PR/8/34 virus NS RNA were mixed with isolated influenza A virus polymerase proteins. This reconstituted ribonucleoprotein (RNP) complex was transfected into MDCK (or 293) cells, which were infected with influenza virus. CAT activity was negligible before and soon after virus infection, but was demonstrable by seven hours post virus infection. When cell supernatant containing budded virus from this "rescue" experiment was used to infect a new monolayer of MDCK cells, CAT activity was also detected, suggesting that the RNA containing the recombinant CAT gene had been packaged into virus particles. These results demonstrate the successful use of recombinant negative strand viral RNA templates and purified RNA-dependent RNA polymerase to reconstitute recombinant influenza virus RNP. Furthermore, the data suggest that the 22 5′ terminal and the 26

3′ terminal sequences of the influenza A virus RNA are sufficient to provide the signals for RNA transcription, A replication and for packaging of RNA into influenza virus particles.

Using this methodology we also demonstrated the rescue of synthetic RNAs, derived from appropriate recombinant plasmid DNAs, into stable and infectious influenza viruses. In particular, RNA corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN) was transcribed in vitro from plasmid DNA and, following the addition of purified influenza virus polymerase complex, was transfected into MDBK cells. Superinfection with helper virus lacking the WSN NA gene resulted in the release of virus containing the WSN NA gene. We then introduced five point mutations into the WSN NA gene by cassette mutagenesis of the plasmid DNA. Sequence analysis of the rescued virus revealed that the genome contained all five mutations present in the mutated plasmid. Similar experiments were carried out using RNA corresponding to the matrix protein (M) gene and using RNA corresponding to the nucleoprotein (NP) gene. This technology can be used to create viruses with site-specific mutations so that influenza viruses with defined biological properties may be engineered. Exemplary M gene mutations are disclosed.

The ability to reconstitute RNPs in vitro allows the design of novel chimeric influenza viruses which express foreign genes. One way to achieve this goal involves modifying existing influenza virus genes. For example, the HA gene may be modified to contain foreign sequences in its external domains. Where the heterologous sequence are epitopes or antigens of pathogens, these chimeric viruses may be used to induce a protective immune response against the disease agent from which these determinants are derived. In addition to modifying genes coding for surface proteins, genes coding for nonsurface proteins may be altered. The latter genes have been shown to be associated with most of he important cellular immune responses in the influenza virus system (Townsend et al., 1985, Cell 42: 475-482). Thus, the inclusion of a foreign determinant in the NP or the NS gene of an influenza virus may - following infection - induce an effective cellular immune response against this determinant. Such an approach may be particularly helpful in situations in which protective immunity heavily depends on the induction of cellular immune responses (e.g., malaria, etc.).

Another approach which would permit the expression of foreign proteins (or domains of such proteins) via chimeric influenza viruses concerns the introduction of complete heterologous genes into the virus. Influenza virus preparations with more than eight RNA segments have previously been described (Nayak, D. et al. in Genetics of Influenza Virus, P. Palese and D.W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255-279). Thus, chimeric influenza viruses with nine or more RNA segments may be viable, and correct packaging of such chimeric viruses may readily occur.

The invention may be divided into the following stages solely for the purpose of description and not by way of limitation: (a) construction of recombinant RNA templates; b) expression of heterologous gene products using the recombinant RNA templates; and (c) rescue of the heterologous gene in recombinant virus particles. For clarity of discussion, the invention is described in the subsections below using influenza. Any strain of influenza (e.g., A, B, C) may be utilized. However, the principles may be analogously applied to construct other negative strand RNA virus templates and chimeric viruses including, but not limited to paramyxoviruses, such as parainfluenza viruses, measles viruses, respiratory syncytial virus; bunyaviruses; arena viruses; etc. A particularly interesting virus system that can be used in accordance with the invention are the orthomyxo-like insect virus called Dhori (Fuller, 1987, Virology 160: 81-87).

a. CONSTRUCTION OF THE RECOMBINANT RNA TEMPLATES

Heterologous gene coding sequences flanked by the complement of the viral polymerase binding site/promoter, e.g, the complement of 3′-influenza virus terminus, or the complements of both the 3′- and 5′-influenza virus termini may be constructed using techniques known in the art. Recombinant DNA molecules containing these hybrid sequences can be cloned and transcribed by a DNA-directed RNA polymerase, such as bacteriophage T7, T3 or the Sp6 polymerase and the like, to produce the recombinant RNA templates which possess the appropriate viral sequences that allow for viral polymerase recognition and activity.

One approach for constructing these hybrid molecules is to insert the heterologous coding sequence into a DNA complement of an influenza virus genomic segment so that the heterologous sequence is flanked by the viral sequences required

for viral polymerase activity; i.e., the viral polymerase binding site/promoter, hereinafter referred to as the viral polymerase binding site. In an alternative approach, oligonucleotides encoding the viral polymerase binding site, e.g., the complement of the 3′-terminus or both termini of the virus genomic segments can be ligated to the heterologous coding sequence to construct the hybrid molecule. The placement of a foreign gene or segment of a foreign gene within a target sequence was formerly dictated by the presence of appropriate restriction enzyme sites within the target sequence. However, recent advances in molecular biology have lessened this problem greatly. Restriction enzyme sites can readily be placed anywhere within a target sequence through the use of site-directed mutagenesis (e.g., see, for example, the techniques described by Kunkel, 1985, Proc. Natl. Acad. Sci. U.S.A. 82;488). Variations in polymerase chain reaction (PCR) technology, described infra, also allow for the specific insertion of sequences (i.e., restriction enzyme sites) and allow for the facile construction of hybrid molecules. Alternatively, PCR reactions could be used to prepare recombinant templates without the need of cloning. For example, PCR reactions could be used to prepare double-stranded DNA molecules containing a DNA-directed RNA polymerase promoter (e.g., bacteriophage T3, T7 or Sp6) and the hybrid sequence containing the heterologous gene and the influenza viral polymerase binding site. RNA templates could then be transcribed directly from this recombinant DNA. In yet another embodiment, the recombinant RNA templates may be prepared by ligating RNAs specifying the negative polarity of the heterologous gene and the vital polymerase binding site using an RNA ligase. Sequence requirements for viral polymerase activity and constructs which may be used in accordance with the invention are described in the subsections below.

i. THE VIRAL 3′-TERMINUS IS REQUIRED FOR POLYMERASE ACTIVITY

The experiments described in Section 6 et seq., infra, are the first to define promoter sequences for a polymerase of a negative-sense RNA virus, and it was found that the specificity lies in the 3′ terminal 15 nucleotides. These viral polymerase binding site sequences, as well as functionally equivalent sequences may be used in accordance with the invention. For example, functionally equivalent sequences containing substitutions, insertions, deletions, additions or inversions which exhibit

similar activity may be utilized. The RNA synthesis by the viral polymerase described infra is a model for specific recognition and elongation by the influenza viral polymerase for the following reasons: (a) the polymerase has high activity when primed with ApG, a feature unique to influenza viral polymerase; (b) it has optimal activity at temperature and ionic conditions previously shown to be effective for the viral RNPs; (c) the polymerase is specific for influenza viral sequences on the model RNA templates; (d) the polymerase is active in the cap-endonuclease primed RNA synthesis which is the hallmark of the influenza viral polymerase; (e) recognition of cap donor RNA is specific to cap 1 structures; and (f) genomic RNA segments are specifically copied.

ii. TERMINAL PANHANDLE IS NOT REQUIRED FOR OPTIMAL RECOGNITION AND SYNTHESIS BY THE VIRAL POLYMERASE

We had previously shown that the influenza vital segment RNAs base-pair at their termini to form panhandle structures. This was achieved by two methods. A cross-linking reagent derivative of psoralen covalently bound the termini of each segment in intact virus or in RNPs from infected cells (Hsu et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). The treated RNA was seen by electron microscopy to be circular, by virtue of the cross-linked termini. Similarly, the RNA termini in RNPs were found to be sensitive to ribonuclease V1, which recognizes and cleaves double-stranded RNA, and the viral polymerase was found to be bound to both termini in the panhandle conformation (Honda, et al., 1988, J. Biochem. 104: 1021-1026). In these studies the panhandle structure of the genomic RNA was shown to exist, and it was inferred to play a role in polymerase recognition. Although the template RNAs used in the examples described, were originally prepared to reveal panhandle-specific protein binding, it was found that the terminal panhandle had no obvious role in the polymerase reactions studied herein.

iii. THE RNA POLYMERASE PREPARATION SPECIFICALLY COPIES NEGATIVE SENSE TEMPLATES

The viral polymerase was shown to synthesize RNA with optimal efficiency if the template had the "wild-type" negative sense 3′ terminus. It was shown that RNAs of unrelated sequence were not copied, and that those with extra polylinker sequences on the 3′ end were much less efficiently copied. A DNA of the correct sequence was

similarly unsuitable as a template. The reaction was highly specific since the M-wt template was replicated only at very low levels. Even though our source of polymerase was intact virus, this finding was very surprising since it had never been suggested that the polymerase which recognizes the viral sense RNA would not efficiently copy the plus sense strand. Studies are underway to examine the specificity of the polymerase purified from infected cells at times post infection when the complementary RNA is copied into genomic templates. The present data support a model whereby the viral polymerase which copies vRNA is functionally different from that which synthesizes vRNA from cRNA by virtue of their promoter recognition. It is possible that by regulated modification of the polymerase in infected cells it then becomes capable of recognizing the 3′ terminus of plus sense RNA. By analyzing promoter mutants we investigated the fine specificity of the reaction and found that the only single mutation which generated a significantly lower level of synthesis was that of V-A_3 RNA. Furthermore, combinations of two or more point changes in positions 3, 5, 8 and 10 greatly lowered synthesis levels.

iv. INSERTION OF THE HETEROLOGOUS GENE SEQUENCE INTO THE PB2 PB1 PA OR NP GENE SEGMENTS

The gene segments coding for the PB2, PB1, PA and NP proteins contain a single open reading frame with 24-45 untranslated nucleotides at their 5′-end, and 22-57 untranslated nucleotides at their 3′-end. Insertion of a foreign gene sequence into any of these segments could be accomplished by either a complete replacement of the viral coding region with the foreign gene or by a partial replacement. Complete replacement would probably best be accomplished through the use of PCR-directed mutagenesis. The principle of this mutagenesis method is illustrated in FIG. 10. Briefly, PCR-primer A would contain, from 5′ to 3′, a unique restriction enzyme site, such as a class IIS restriction enzyme site (i.e., a "shifter" enzyme; that recognizes a specific sequence but cleaves the DNA either upstream or downstream of that sequence); the entire 3′ untranslated region of the influenza gene segment; and a stretch of nucleotides complementary to the carboxy-terminus coding portion of the foreign gene product. PCR-primer B would contain from the 5′ to 3′ end: a unique restriction enzyme site; a truncated but active phage polymerase sequence; the complement of the entire 5′ untranslated region of the influenza gene segment (with respect to the

negative sense vRNA); and a stretch of nucleotides corresponding to the 5′ coding portion of the foreign gene. After a PCR reaction using these primers with a cloned copy of the foreign gene, the product may be excised and cloned using the unique restriction sites. Digestion with the class IIS enzyme and transcription with the purified phage polymerase would generate an RNA molecule containing the exact untranslated ends of the influenza viral gene segment with a foreign gene insertion. Such a construction is described for the chloramphenicol acetyltransferase (CAT) gene used in the examples described in Section 7 infra. In an alternate embodiment, PCR-primed reactions could be used to prepare double-stranded DNA containing the bacteriophage promoter sequence, and the hybrid gene sequence so that RNA templates an be transcribed directly without cloning.

Depending on the integrity of the foreign gene product and the purpose of the construction, it may be desirable to construct hybrid sequences that will direct the expression of fusion proteins. For example, the four influenza virus proteins, PB2, PBI, PA or NP are polymerase proteins which are directed to the nucleus of the infected cell through specific sequences present in the protein. For the NP this amino acid sequence has been found to be (single letter code) QLVWMACNSAAFEDLRVLS (Davey et al., 1985, Cell 40: 667-675). Therefore, if it is desired to direct the foreign gene product to the nucleus (if by itself it would not ordinarily do so) the hybrid protein should be engineered to contain a domain which directs it there. This domain could be of influenza viral origin, but not necessarily so. Hybrid proteins can also be made from non-viral sources, as long as they contain the necessary sequences for replication by influenza virus (3′ untranslated region, etc.). As another example, certain antigenic regions of the viral gene products may be substituted with foreign sequences. Townsend et al., (1985, Cell 42: 475-482), identified an epitope within the NP molecule which is able to elicit a vigorous CTL (cytotoxic T cell) response. This epitope spans residues 147-161 of the NP protein and consists of the amino acids TYQRTRQLVRLTGMDP. Substituting a short foreign epitope in place of this NP sequence may elicit a strong cellular immune response against the intact foreign antigen. Conversely, expression of a foreign gene product containing this 15 amino acid region may also help induce a strong cellular immune response against the foreign protein.

v. INSERTION OF THE HETEROLOGOUS GENE SEQUENCE INTO THE HA OR NA GENE SEGMENTS

The HA and NA proteins, coded for by separate gene segments, are the major surface glycoproteins of the virus. Consequently, these proteins are the major targets for the humoral immune response after infection. They have been the most widely-studied of all the influenza viral proteins as the three-dimensional structures of both these proteins have been solved.

The three-dimensional structure of the H3 hemagglutinin along with sequence information on large numbers of variants has allowed for the elucidation of the antigenic sites on the HA molecule (Webster et al., 1983, In Genetics Of Influenza Virus, P. Palese and D.W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 127-160). These sites fall into four discrete non-overlapping regions on the surface of the HA. These regions are highly variable and have also been shown to be able to accept insertions and deletions. Therefore, substitution of these sites within HA (e.g., site A; amino acids 122-147 of the A/HK/68 HA) with a portion of a foreign protein may provide for a vigorous humoral response against this foreign peptide. In a different approach, the foreign peptide sequence may be inserted within the antigenic site without deleting any viral sequences. Expression products of such constructs may be useful in vaccines against the foreign antigen, and may indeed circumvent a problem discussed earlier, that of propagation of the recombinant virus in the vaccinated host. An intact HA molecule with a substitution only in antigenic sites may allow for HA function and thus allow for the construction of a viable virus. Therefore, this virus can be grown without the need for additional helper functions. Of course, the virus should be attenuated in other ways to avoid any danger of accidental escape.

Other hybrid constructions may be made to express proteins on the cell surface or enable them to be released from the cell. As a surface glycoprotein, the HA has an amino-terminal cleavable signal sequence necessary for transport to the cell surface, and a carboxy-terminal sequence necessary for membrane anchoring. In order to express an intact foreign protein on the cell surface it may be necessary to use these HA signals to create a hybrid protein. Alternatively, if only the transport signals are present and the membrane anchoring domain is absent, the protein may be excreted out of the cell.

In the case of the NA protein, the three-dimensional structure is known but the antigenic sites are spread out over the surface of the molecule and are overlapping. This indicates that if a sequence is inserted within the NA molecule and it is expressed on the outside surface of the NA it will be immunogenic. Additionally, as a surface glycoprotein, the NA exhibits two striking differences from the HA protein. Firstly, the NA does not contain a cleavable signal sequence; in fact, the amino-terminal signal sequence acts as a membrane anchoring domain. The consequence of this, and the second difference between the NA and HA, is that the NA is orientated with the amino-terminus in the membrane while the HA is orientated with the carboxy-terminus in the membrane. Therefore it may be advantageous in some cases to construct a hybrid NA protein, since the fusion protein will be orientated opposite of a HA-fusion hybrid.

vi. INSERTION OF THE HETEROLOGOUS GENE INTO THE NS AND M GENE SEGMENTS

The unique property of the NS and M segments as compared to the other six gene segments of influenza virus is that these segments code for at least two protein products. In each case, one protein is coded for by an mRNA which is co-linear with genomic RNA while the other protein is coded for by a spliced message. However, since the splice donor site occurs within the coding region for the co-linear transcript, the NS1 and NS2 proteins have an identical 10 amino acid amino terminus while MI and M2 have an identical 14 amino acid amino terminus.

As a result of this unique structure, recombinant viruses may be constructed so as to replace one gene product within the segment while leaving the second product intact. For instance, replacement of the bulk of the NS2 or M2 coding region with a foreign gene product (keeping the splice acceptor site) could result in the expression of an intact NS1 or M1 protein and a fusion protein instead of NS2 or M2. Alternatively, a foreign gene may be inserted within the NS gene segment without affecting either NS1 or NS2 expression. Although most NS genes contain a substantial overlap of NS1 and NS2 reading frames, certain natural NS genes do not. We have analyzed the NS gene segment from A/Ty/Or/71 virus (Norton et al., 1987, Virology 156: 204-213) and found that in this particular gene, the NS1 protein terminates at nucleotide position 409 of the NS gene segment while the splice acceptor site for the

NS2 is at nucleotide position 528. Therefore, a foreign gene could be placed between the termination codon of the NS1 coding region and the splice acceptor site of the NS2 coding region without affecting either protein. It may be necessary to include a splice acceptor site at the 5′ end of the foreign gene sequence to ensure protein production (this would encode a hybrid protein containing the amino-terminus of NS1). In this way, the recombinant virus should not be defective and should be able to be propagated without need of helper functions.

Although the influenza virus genome consists of eight functional gene segments it is unknown how many actual segments a virus packages. It has been suggested that influenza can package more than eight segments, and possibly up to 12 (Lamb and Choppin, 1983, Ann. Rev. Biochem. 52: 467-506). This would allow for easier propagation of recombinant virus in that "ninth" gene segment could be designed to express the foreign gene product. Although this "ninth" segment may be incorporated into some viruses, it would soon be lost during virus growth unless some selection is supplied. This can be accomplished by "uncoupling" the NS or M gene segment. The NS2 coding portion could be removed from the NS gene segment and placed on the gene segment coding for the foreign protein (along with appropriate splicing signals). Alternatively, a bicistronic mRNA could be constructed to permit internal initiation to "unsplice" these viral sequences; for example, using the sequences described by Pelletier et al., 1988, Nature 334: 320-325. The resulting recombinant virus with the "uncoupled" NS or M gene would be able to propagate on its own and also would necessarily have to package the "ninth" gene segment, thus ensuring expression of the foreign gene.

b. EXPRESSION OF HETEROLOGOUS GENE PRODUCTS USING RECOMBINANT RNA TEMPLATE

The recombinant templates prepared as described above can be used in a variety of ways to express the heterologous gene products in appropriate host cells or to create chimeric viruses that express the heterologous gene products. In one embodiment, the recombinant template can be combined with viral polymerase complex purified as described in Section 6, infra, to produce rRNPs which are infectious. Alternatively, the recombinant template may be mixed with viral polymerase complex prepared using recombinant DNA methods (e.g., see Kingsbury et al., 1987, Virology 156: 396-403).

Such rRNPs, when used to transfect appropriate host cells, may direct the expression of the heterologous gene product at high levels. Host cell systems which provide for high levels of expression include continuous cell lines that supply viral functions such as cell lines superinfected with influenza, cell lines engineered to complement influenza viral functions, etc.

In an alternate embodiment of the invention, the recombinant templates or the rRNPs may be used to transfect cell lines that express the viral polymerase proteins in order to achieve expression of the heterologous gene product. To this end, transformed cell lines that express all three polymerase proteins such as 3P-38 and 3P-133 (Krystal et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83: 2709-2713) may be utilized as appropriate host cells. Host cells may be similarly engineered to provide other viral functions or additional functions such as NP.

i. PURIFICATION OF THE VIRAL POLYMERASE

The viral polymerase proteins used to produce the rRNPs may be purified from dissociated RNP cores isolated from whole virus. In general, RNP cores may be prepared using standard methods (Plotch et al., 1981, Cell 23: 847-858; Rochavansky, 1976, Virology 73: 327-338). The pooled RNP cores may then be centrifuged on a second gradient of CsCl (1.5-3.0 M) and glycerol (30%-45%) as described by Honda et al., 1988, J. Biochem. 104: 1021-1026. The active viral polymerase fractions may be isolated from top of the gradient, i.e., in the region of the gradient correlating with 1.5 to 2.0 M CsCl and corresponding to the fraction Honda et al. identified as "NP". Surprisingly, this fraction contains all the viral polymerase proteins required for the active complex. Moreover, the P proteins which may be recovered from the bottom of the gradient are not required, and indeed do not provide for the transcription of full length viral RNA. Thus, it appears that the so-called "NP" fraction contains, in addition to NP, the active forms of the PB2, PB1, and PA proteins.

ii. HIGH CONCENTRATIONS OF POLYMERASE ARE REQUIRED FOR CAP-PRIMED RNA SYNTHESIS

High concentrations of viral polymerase complex are able to catalyze this virus-specific cap-endonuclease primed transcription. Under the conditions specified in Section 6 infra, about 50 ng NP with 200 pg of the three P proteins were found to react optimally with 5 to 10 ng RNA reaction. The observation has been that although

the NP selectively encapsidates influenza vRNA or cRNA in vivo, the NP will bind to RNA nonspecifically in vitro (Kingsbury, et al., 1987, Virology 156: 396-403; Scholtissek and Becht, 1971, J. Gen. Virol. 10: 11-16). Presumably, in order for the viral polymerase to recognize the viral template RNAs in our in vitro reaction, they have to be encapsidated by the NP. Therefore, the addition of a capped mRNA primer would essentially compete with the template RNA for binding of NP. Since the dinucleotide ApG would not be expected to bind NP, the low concentration polymerase was able to use only the short templates with ApG. Supporting this hypothesis is the observation that the higher concentration polymerase preparation is inhibited through the addition of progressively higher amounts of either template RNA or any non-specific RNA. It should also be noted that the unusual specificity for the m7GpppXm cap 1 structure previously shown with viral RNPs was also found with the reconstituted RNPs.

iii. GENOMIC LENGTH RNA TEMPLATES ARE EFFICIENTLY COPIED

Plasmid-derived RNA identical to segment 8 of the A/WSN/33 virus was specifically copied by the polymerase (using the PCR method described in FIG. 10). In reactions using RNA extracted from virus, all eight segments were copied, although the HA gene was copied at a lower level. The background in these reactions was decreased in comparison to the 30 to 53 nt templates, probably since the contaminating RNAs in the polymerase preparation were predominantly defective RNAs of small size. Recombinant templates encoding foreign genes transcribed in this system may be used to rescue the engineered gene in a virus particle.

c. PREPARATION OF CHIMERIC NEGATIVE STRAND RNA VIRUS

In order to prepare chimeric virus, reconstituted RNPs containing modified influenza virus RNAs or RNA coding for foreign proteins may be used to transfect cells which are also infected with a "parent" influenza virus. Alternatively, the reconstituted RNP preparations may be mixed with the RNPs of wild type parent virus and used for transfection directly. Following reassortment, the novel viruses may be isolated and their genomes be identified through hybridization analysis. In additional approaches described herein for the production of infectious chimeric virus, rRNPs may be replicated in host cell systems that express the influenza viral polymerase

proteins (e.g., in virus/host cell expression systems; transformed cell lines engineered to express the polymerase proteins, etc.), so that infectious chimeric virus are rescued; in this instance, helper virus need not be utilized since this function is provided by the viral polymerase proteins expressed. In a particularly desirable approach, cells infected with rRNPs engineered for all eight influenza virus segments may result in the production of infectious chimeric virus which contain the desired genotype; thus eliminating the need for a selection system.

Theoretically, one can replace any one of the eight gene segments, or part of any one of the eight segments with the foreign sequence. However, a necessary part of this equation is the ability to propagate the defective virus (defective because a normal viral gene product is missing or altered). A number of possible approaches exist to circumvent this problem. We have shown that mutants of influenza virus defective in the PB2 and NP proteins can be grown to substantially higher titers in cell lines which were constructed to constitutively express the polymerase and NP proteins (Krystal et al., 1986 Proc. Natl. Acad. Sci. U.S.A. 83: 2709-2813). Similar techniques may be used to construct transformed cell lines that constitutively express any of the influenza genes. These cell lines which are made to express the viral protein may be used to complement the defect in the recombinant virus and thereby propagate it. Alternatively, certain natural host range systems may be available to propagate recombinant virus. An example of this approach concerns the natural influenza isolate CR43-3. This virus will grow normally when passaged in primary chick kidney cells (PCK) but will not grow in Madin-Darby canine kidney cells (MDCK), a natural host for influenza (Maassab & DeBorde, 1983, Virology 130: 342-350). When we analyzed this virus we found that it codes for a defective NS1 protein caused by a deletion of 12 amino acids. The PCK cells contain some activity which either complements the defective NS1 protein or can completely substitute for the defective protein.

A third approach to propagating the recombinant virus may involve co-cultivation with wild-type virus. This could be done by simply taking recombinant virus and co-infecting cells with this and another wild-type virus (preferably a vaccine strain). The wild-type virus should complement for the defective virus gene product and allow growth of both the wild-type and recombinant virus. This would be an

analogous situation to the propagation of defective-interfering particles of influenza virus (Nayak et al., 1983, In: Genetics of Influenza Viruses, P. Palese and D.W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255-279). In the case of defective-interfering viruses, conditions can be modified such that the majority of the propagated virus is the defective particle rather than the wild-type virus. Therefore this approach may be useful in generating high titer stocks of recombinant virus. However, these stocks would necessarily contain some wild-type virus.

Alternatively, synthetic RNPs may be replicated in cells co-infected with recombinant viruses that express the influenza virus polymerase proteins. In fact, this method may be used to rescue recombinant infectious virus in accordance with the invention. To this end, the influenza virus polymerase proteins may be expressed in any expression vector/host cell system, including but not limited to viral expression vectors (e.g., vaccinia virus, adenovirus, baculovirus, etc.) or cell lines that express the polymerase proteins (e.g., see Krystal et al., 1986, Proc. Natl. Acad. Sci. USA 83: 2709-2713). Moreover, infection of host cells with rRNPs encoding all eight influenza virus proteins may result in the production of infectious chimeric virus particles. This system would eliminate the need for a selection system, as all recombinant virus produced would be of the desired genotype. In the examples herein, we describe a completely synthetic replication system where, rather than infecting cells with influenza virus, synthetic RNP's are replicated in cells through the action of influenza virus proteins expressed by recombinant vaccinia vectors. In this way we show that the only influenza virus proteins essential for transcription and replication of RNP are the three polymerase proteins and the nucleoprotein.

It should be noted that it may be possible to construct a recombinant virus without altering virus viability. These altered viruses would then be growth competent and would not need helper functions to replicate. For example, alterations in the hemagglutinin gene segment and the NS gene segment discussed, supra, may be used to construct such viable chimeric viruses.

In the examples infra, the construction of a recombinant plasmid is described that, following transcription by T7 polymerase, yielded an RNA template which was recognized and transcribed by the influenza virus polymerase in vitro. This RNA template corresponds to the NS RNA of an influenza virus except that the viral coding

sequences are replaced by those of a CAT gene. This recombinant negative strand viral RNA template was then mixed with purified influenza virus polymerase to reconstitute an RNP complex. The recombinant RNP complex was transfected into cells which were then infected with influenza virus, leading to expression of CAT activity.

A number of factors indicate that this system represents a biologically active recombinant RNP complex which is under tight control of the signals for transcription, replication and packaging of influenza virus RNAs. First, the CAT gene is of negative polarity in the recombinant viral RNA used for RNP transfection. Thus, the incoming RNA cannot be translated directly in the cell and must first be transcribed by the influenza virus polymerase to permit translation and expression of the CAT gene. Secondly, neither transfected naked recombinant RNA alone in the presence of infecting helper virus, nor recombinant RNP complex in the absence of infecting helper virus is successful in inducing CAT activity. This suggests that influenza viral proteins provided by the incoming RNP, as well as by the infecting helper virus, are necessary for the amplification of the recombinant RNA template. Finally, after RNP-transfection and infection by helper virus, virus particles emerge which apparently contain the recombinant RNA, since these particles again induce CAT activity in freshly infected cells. These results suggest that the 26 3′ terminal and the 22 5′ terminal nucleotides corresponding to the terminal nucleotides in the influenza A virus NS RNA are sufficient to provide the signals for polymerase transcription and replication, as well as for packaging of the RNA into particles.

The foregoing results, which defined the cis acting sequences required for transcription, replication and packaging of influenza virus RNAs, were extended by additional working examples, described infra, which demonstrate that recombinant DNA techniques can be used to introduce site-specific mutations into the genomes of infectious influenza viruses.

Synthetic RNAs, derived by transcription of plasmid RNA in vitro were used in RNP-transfection experiments to rescue infectious influenza virus. To enable selection of this virus, we chose a system that required the presence of a WSN-like neuraminidase gene in the rescued virus. Viruses containing this gene can grow in MDBK cells in the absence of protease in the medium (Schulman et al., 1977, J. Virol.

24: 170-176). The helper virus WSN-HK does not grow under these circumstances. Clearly, alternative selection systems exist. For example, antibody screens or conditionally lethal mutants could be used to isolate rescued viruses containing RNAs derived from plasmid DNAs. In the experiments described infra, viruses which were WSN virus-like were recovered. The WSN NA gene was derived from plasmid DNAs or from purified WSN virion RNA (FIG. 17, lanes 2 and 5). In the latter case, using whole virion RNA for the RNP-transfection, we do not know whether other genes were also transferred to the rescued virus, since the helper virus shares the remaining seven genes with WSN virus. The rescued viruses had the expected RNA patterns (FIG. 17) and grew to titers in MDBK or MDCK cells which were indistinguishable from those of the wild type WSN virus. It should be noted that rescue of an NA RNA containing a single nucleotide deletion in the 5′ nontranslated region was not possible. This again illustrates the importance of regulatory sequences present in the non-translated regions of influenza virus RNAs. We also rescued virus using RNA that was engineered to contain 5 nucleotide changes in a 39 nucleotide long region (FIG. 16). We verified the presence of these mutations in the rescued mutant virus by direct sequencing of the RNA (FIG. 18). These mutations did not result in any amino acid change in the neuraminidase protein and thus were not expected to change the biological property of the virus. Although this virus was not extensively studied, its plaquing behavior and its growth characteristics were indistinguishable from that of wild type WSN virus. Similarly, the WSN NP gene, the WSN M gene and the PR8 M gene were derived from plasmid DNAs and rescued using temperature sensitive mutants of WSN/HK as helper viruses. Using such technology, mutations may be introduced that will change the biological characteristics of influenza viruses. For example, we rescued virus using M gene RNA that was engineered to contain a single nucleotide change. We verified the presence of the mutation in the rescued mutant virus by direct sequencing. These studies will help in distinguishing the precise functions of all the viral proteins, including those of the nonstructural proteins. In addition, the nontranslated regions of the genome can be studied by mutagenesis, which should lead to a better understanding of the regulatory signals present in viral RNAs. An additional area of great interest concerns the development of the influenza virus system as a vaccine vector.

d. VACCINE FORMULATIONS USING THE CHIMERIC VIRUSES

Virtually any heterologous gene sequence may be constructed into the chimeric viruses of the invention for use in vaccines. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as part of the chimeric viruses. For example, heterologous gene sequences that can be constructed into the chimeric viruses of the invention for use in vaccines include but are not limited to epitopes of human immunodeficiency virus (HIV) such as gp120; hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g., gD, gE); VP1 of poliovirus; antigenic determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of anti-idiotypic immunoglobulins that mimic such epitopes may be constructed into the chimeric viruses of the invention.

Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification.

In this regard, the use of genetically engineered influenza virus (vectors) for vaccine purposes may require the presence of attenuation characteristics in these strains. Current live virus vaccine candidates for use in humans are either cold adapted, temperature sensitive, or passaged so that they derive several (six) genes from avian viruses, which results in attenuation. The introduction of appropriate mutations (e.g., deletions) into the templates used for transfection may produce the novel viruses with attenuation characteristics. For example, specific missense mutations which are associated with temperature sensitivity or cold adaptation can be made into deletion mutations. These mutations should be more stable than the point mutations associated with cold or temperature sensitive mutants and reversion frequencies should be extremely low.

Alternatively, chimeric viruses with "suicide" characteristics may be constructed. Such viruses would go through only one or a few rounds of replication in the host. For example, cleavage of the HA is necessary to allow for reinitiation of replication. Therefore, changes in the HA cleavage site may provide a virus that replicates in an appropriate cell system but not in the human host. When used as a vaccine, the recombinant virus would go through a single replication cycle and induce a sufficient level of immune response but it would not go further in the human host and cause disease. Recombinant viruses lacking one or more of the essential influenza virus genes would not be able to undergo successive rounds of replication. Such defective viruses can be produced by co-transfecting reconstituted RNPs lacking a specific gene(s) into cell lines which permanently express this gene(s). Viruses lacking an essential gene(s) will be replicated in these cell lines but when administered to the human host will not be able to complete a round of replication. Such preparations may transcribe and translate -- in this abortive cycle -- a sufficient number of genes to induce an immune response. Alternatively, larger quantities of the strains could be administered, so that these preparations serve as inactivated (killed) virus vaccines. For inactivated vaccines, it is preferred that the heterologous gene product be expressed as a viral component, so that the gene product is associated with the virion. The advantage of such preparations is that they contain native proteins and do not undergo inactivation by treatment with formalin or other agents used in the manufacturing of killed virus vaccines.

In another embodiment of this aspect of the invention, inactivated vaccine formulations may be prepared using conventional techniques to "kill" the chimeric viruses. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity of the virus is destroyed without affecting its immunogenicity. In order to prepare inactivated vaccines, the chimeric virus may be grown in cell culture or in the allantois of the chick embryo, purified by zonal ultracentrifugation, inactivated by formaldehyde or β-propiolactone, and pooled. The resulting vaccine is usually inoculated intramuscularly.

Inactivated viruses may be formulated with a suitable adjuvant in order to enhance the immunological response. Such adjuvants may include but are not limited to mineral gels, e.g., aluminum hydroxide; surface active substances such as

lysolecithin, pluronic polyols, polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG and Corynebacterium parvum.

Many methods may be used to introduce the vaccine formulations described above. These include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be preferable to introduce the chimeric virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed. Where a live chimeric virus vaccine preparation is used, it may be preferable to introduce the formulation via the natural route of infection for influenza virus. The ability of influenza virus to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by chimeric influenza viruses may induce a strong secretory immune response, for example in the urogenital system, with concomitant protection against a particular disease causing agent.

5. EXAMPLE: PROMOTER ANALYSIS OF THE INFLUENZA VIRAL RNA POLYMERASE

In the examples described below, polymerase which is depleted of genomic RNA was prepared from the upper fractions of the CsCl-glycerol gradient centrifugation. This polymerase is able to copy short model templates which are derived from transcription of appropriate plasmic DNA with bacteriophage T7 RNA polymerase in a sequence-specific manner. The termini of this model RNA are identical to the 3′ 15 and 5′ 22 nucleotides conserved in segment 8 from all influenza A viral RNAs. By manipulating the plasmid in order to prepare different RNAs to serve as template, we demonstrated that recognition of and synthesis from this model RNA was specific for the promoter at the 3′ terminal sequence and did not require the panhandle. In addition, site specific mutagenesis identified nucleotide positions responsible for the viral polymerase favoring synthesis from genomic sense templates over complementary sense RNA. Conditions were also found in which cap-endonuclease primed RNA synthesis could be observed using model RNAs. In addition, the reconstituted system permitted virus-specific synthesis from genomic length RNAs, derived either from plasmids or from RNA purified from virus through phenol extraction.

a. MATERIALS AND METHODS

i. PURIFICATION OF THE VIRAL RNA POLYMERASE

RNP cores were prepared from whole virus using standard methods (Plotch, et al., 1981, Cell 23: 847-858; Rochavansky, 1976, Virology 73: 327-338). Two to three milligrams of virus were disrupted by incubating in 1.5% Triton N-101, 10 mg/ml lysolecithin, 100 mM tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol and 1.5 mM dithiothreitol. The sample was fractionated by centrifugation on a 30-70% glycerol (w/v) step gradient in the presence of 50 mM tris-HCl, pH 7.8 and 150 mM NaCl. The core preparation was centrifuged at 45,000 rpm in an SW50.1 rotor for 4 hours at 4°C. Fractions enriched in RNP were identified by SDS-polyacrylamide gel electrophoresis of protein samples from each fraction and staining with silver. The core fractions were then subjected to a second gradient centrifugation as was described in Honda et al. 1988, J. Biochem. 104: 1021-1026. This second gradient had steps of 0.5 ml 3.0 M CsCl and 45% (w/v) glycerol, 1.75 ml 2.5 M CsCl and 40% glycerol, 1.25 ml 2.0 M CsCl and 35% glycerol, and 1.0 ml of 1.5 M CsCl and 30% glycerol. All steps were buffered with 50 mM tris-HCl, pH 7.6 and 100 mM NaCl. 0.5 ml of RNP cores were layered on top and the sample was centrifuged at 45,000 rpm in an SW50.1 rotor for 25 hours at 4°C. Polymerase fractions were again identified by SDS-polyacrylamide electrophoresis of the protein samples and silver staining. Active polymerase fractions were generally found in the region of the gradient correlating with 1.5 to 2.0 M CsCl. These fractions were pooled and then dialyzed against 50 mM tri-HCl, pH 7.6, 100 mM NaCl and 10 mM $MgCl_2$, and concentrated in centricon-10 tubes (Amicon) or fractions were dialyzed in bags against 50 mM tris-HCl, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 50% glycerol.

ii. PREPARATION OF PLASMID

The plasmid design is indicated in FIG. 2. Insert DNA for the pV-wt plasmid was prepared using an Applied Biosystems DNA synthesizer. The "top" strand was 5'-GAAGCTTAATACGACTCACTATAAGTAGAAACAAGGGTGTTTTTTCATATC ATTTAAACTTCACCCTGCTTTTGCTGAATTCATTCTTCTGCAGG-3'. The "bottom" strand was synthesized by primer-extension with 5'-CCTGCAGAAGAATGA-3' as primer. The 95 bp DNA was digested with HindIII and PstI and purified by

extraction with phenol/chloroform, ethanol precipitation, and passage over a NACS-prepack ion Exchange column (Bethesda Research Laboratories). This DNA was ligated into pUC-19 which had been digested with HindIII and PstI and then used to transform E. coli strain DH5-α which had been made competent using standard protocols. Bacteria were spread on agar plates containing X-gal and IPTG, and blue colonies were found to have the plasmid containing the predicted insert since the small insert conserved the lacZ reading frame and did not contain a termination codon. The pM-wt plasmid was prepared by a similar strategy except that both strands were chemically synthesized with the upper strand having the sequence 5'-GAAGCTTAATACGACTCACTATAAGCAAAAGCAGGGTGAAGTTTAAATGATAT-GAAAAAACACCCTTGTTTCTACTGAATTCATTCTTCTGCAGG-3'.

The pV-d5' plasmid (FIG. 2) was prepared using the oligonucleotides 5'-AGCTTAATACGACTCACTATAAGATCTATTAAACT-TCACCCTGCTTTTGCTGAATTCATTCTTCTGCA-3' and 5'-GAAGAATGAAT-TCAGCAAAAGCAGGGTGAAGTTTAATAGATCTTATAGTGAGTCGTATTA-3'. The DNAs were annealed and ligated into the HindIII/PstI digested pUC-19 and white colonies were found to contain the correct plasmid because this insert resulted in a frameshift in the lacZ gene. The point mutants were isolated following digestion of pV-d5' with BglII and PstI and ligation of the linearized plasmid with a single stranded oligonucleotide of mixed composition. Since BglII laves a 5' extension and PstI a 3' extension, a single oligonucleotide was all that was necessary for ligation of insert. The host cell was then able to repair gaps caused by the lack of a complementary oligonucleotide. Oligonucleotides were designed to repair the frameshift in the lacZ gene so that bacteria which contained mutant plasmids were selected by their blue color.

Plasmid pHgaNS, which was used to prepare an RNA identical to segment 8 of A/WSN/33, was prepared using the primers 5'-CCGAATTCTTAATACGACTCACTATAAGTAGAAACAAGGGTG-3' and 5'-CCTCTAGACGCTCGAGAGCAAAAGCAGGTG-3' in a polymerase chain reaction off of a cDNA clone. The product was then cloned into the XbaI/EcoRI window of pUC19.

iii. PREPARATION OF RNA TEMPLATES

Plasmid DNAs were digested with MboII or other appropriate endonucleases (see FIG.2), and the linearized DNA was transcribed using the bacteriophage T7 RNA polymerase. Run-off RNA transcripts were treated with RNAse-free DNAse 1 and then the RNA was purified from the proteins and free nucleotides using Qiagen tip-5 ion exchange columns (Qiagen, Inc.). Following precipitation in ethanol, purified RNAs were resuspended in water and a sample was analyzed by electrophoresis and followed by silver staining of the polyacrylamide gel in order to quantify the yield of RNA.

iv. INFLUENZA VIRAL POLYMERASE REACTIONS

In a 25 μl total volume, about 30 μg of nucleoprotein and 200 pg total of the three polymerase proteins were mixed with 10 ng of template RNA and the solution was made up to a final concentration of: 50 mM Hepes pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% NP-40, 0.4 mM adenylyl-(3'-5')-guanosyl (ApG) dinucleotide (Pharmacia), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and approximately 0.6 μM α-^{32}P-UTP (40 μCi at 3000 Ci/mmole, New England Nuclear). Reactions were assembled on ice and then transferred to a 30°C water bath for 90 minutes. Reactions were terminated by the addition of 0.18 ml ice-cold 0.3 M sodium acetate/10 mM EDTA and were then extracted with phenol/chloroform (1:1 volume ratio). Following the first extraction, 15 μg polyI-polyC RNA was added as carrier, and the sample was extracted again with phenol/chloroform. The samples were then extracted with ether and precipitated in ethanol. Following centrifugation, the RNA pellet was washed twice with 70% ethanol and then dried under vacuum.

In reactions using the high concentration polymerase, conditions were identical as above except that 20 ng of template RNA were added. In reactions using genomic length RNAs, the amount of polymerase used was doubled, 50 ng of template RNA was used, and the UTP concentration was raised to 2.6 μM.

The RNA was resuspended in a dye mix containing 78% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.05% bromophenol blue. Typically, a sample from this RNA was electrophoresed on an 8% polyacrylamide gel in the absence of urea, and the remainder was denatured by heating to 100°C for 1.5 minutes and an aliquot was loaded on an 8% polyacrylamide gel containing 7.7 M urea. Gels were fixed by

a two step procedure, first in 10% acetic acid, and then in 25% methanol/8% acetic acid. Gels were dried onto filter paper and then exposed to x-ray film.

When different RNAs were being tested for use as template, the different RNA preparations were always analyzed on polyacrylamide gels and stained with silver in order that equal amounts of each template were used. To quantitate the amount of product, gels were exposed to x-ray film in the absence of an intensifying screen in order to improve the linearity of the densitometer readings. Autoradiographs were analyzed using a FB910 scanning densitometer (Fisher Biotech) and peaks were evaluated using computer software from Fisher Biotech.

v. NUCLEASE ANALYSIS OF REACTION PRODUCTS

For ribonuclease T1 analysis of the two principle RNA products, reaction products were analyzed by 8% polyacrylamide gel electrophoresis (without urea) and the gel was not treated with fixative. The wet gel was exposed to an x-ray film and the appropriate gel pieces were located and excised. The gel piece was crushed in 0.3 ml containing 10 mM tris pH 7.5, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 1 μg tRNA as carrier. The RNA diffused into this solution for 3 hours and then the gel was pelleted and the supernatant was made 0.3M in sodium acetate. The supernatant was then extracted twice in phenol/chloroform and once in ether and then precipitated in ethanol. The RNA pellet was resuspended in 5 μl formamide, denatured in boiling water for 1.5 minutes and then diluted by the addition of 0.1 ml 10 mM tris-HCl, pH 7.5, and 1 mM EDTA. Ribonuclease T1 (50 units, Boehringer Mannheim Biochemicals) was added and the samples were incubated for 60 minutes at 37°C. V-wt and M-wt RNAs synthesized with T7 RNA polymerase in the presence of α-^{32}P-UTP were similarly digested with RNAse T1. Reaction products were extracted in phenol/chloroform and precipitated in ethanol and then were analyzed on 20% polyacrylamide gels containing 7.7 M urea.

Nuclease S1 analysis of reaction products was done on transcribed RNA by first terminating the standard polymerase reaction through the addition of S1 buffer to a volume of 0.2 ml with 0.26 M NaCl, 0.05 M sodium acetate, pH 4.6, and 4.5 mM zinc sulfate. The sample was divided into two 0.1 ml volumes and 100 units of S1 nuclease (Sigma Chemical Company) were added to one tube. The samples were incubated for 60 minutes at 37°C. Following the incubation, EDTA (10 mM final

concentration) and 15 μg polyI-polyC RNA was added and the sample was extracted with phenol/chloroform and precipitated in ethanol. The samples were then subjected to polyacrylamide gel electrophoresis.

b. RESULTS

i. PREPARATION OF INFLUENZA VIRAL RNA POLYMERASE AND OF TEMPLATE RNA

RNP cores of influenza virus A/Puerto Rico/8/34 were prepared by disruption of virus in lysolecithin and Triton N-101 followed by glycerol gradient centrifugation (Rochavansky, 1976, Virology 73: 327-338). Fractions containing cores were then subjected to a second centrifugation in a CsCl-glycerol step gradient (Honda, et al., 1988, J. Biochem. 104: 1021-1026). Fractions containing the polymerase were identified by gel electrophoresis of samples followed by silver-staining. Fig. 1 shows the polymerase preparation after CsCl centrifugation. Bovine serum albumin (BSA) was added during dialysis to protect against protein loss. Densitometric scanning of lane 4 compared to known quantities of whole virus in lanes 1 and 2 allowed us to estimate that the proteins in lane 4 consist of 150 ng of NP and about 1 ng total of the three polymerase proteins. One fifth of the preparation used for this gel was used per reaction.

The overall design of the plasmids used to prepare template RNAs in this study is depicted in Figure 2. The entire insert was prepared using oligonucleotides from a DNA synthesizer which were then cloned into the polylinker of pUC19. The insert contained a truncated promoter sequence recognized by the bacteriophage T7 RNA polymerase (Studier and Dunn, 1983, Cold Spring Harbor Symposia on Quantitative Biology, XLVII, 999-1007) so that the first nucleotides synthesized were the terminal 22 nucleotides (nt) of the conserved sequence from the 5′ end of the genome RNA. When the plasmid was cut with restriction endonuclease MboII (which cuts 7 bases upstream of its recognition site), the RNA which resulted from T7 RNA polymerase transcription ended with the terminal 3′ nucleotides of the influenza viral sequence. Included in the sequence was the poly-U stretch adjacent to the 5′ end of the conserved terminus which is thought to comprise at least part of the termination-polyadenylation signal (Robertson, et al., 1981, J. Virol. 38, 157-163). The total length of this model genomic RNA was 53 nt since a 16 nt spacer separated the terminal conserved

sequences. The model RNA which contained both termini identical to those of vRNA was named V-wt. The RNA M-wt encoded the exact complementary strand of V-wt so that the termini match those of complementary RNA (cRNA). V-wt and M-wt were constructed to serve as models for influenza virus-specific vRNA and cRNA, respectively.

ii. VIRAL POLYMERASE CATALYZES SYNTHESIS OF A FULL LENGTH COPY OF THE TEMPLATE

In the reaction using the influenza viral polymerase, V-wt template and ApG primer, a product was obtained which comigrated with a 53 nt RNA on denaturing gels. RNA migrating as a doublet at a position of about 40 to 45 nucleotides (FIG. 3A, lane 2) was also seen. This shorter product is shown below to be RNA which had terminated at a stretch of adenosines present between nucleotides 43-48 in the virion sense template. In addition to the template specific transcripts, a general background of light bands could be seen which correspond to truncated RNA products transcribed from viral genomic RNA not removed during the CsCl-glycerol centrifugation step. When no primer is used, there was no specific transcription product seen (FIG. 3A, lane 3). Additional experiments showed globin mRNA, containing a terminal cap 1 structure, was inactive as primer using initial preparations of polymerase.

When the polymerase reaction was terminated by the addition of excess buffer favorable for nuclease S1 digestion and nuclease was added, the radioactively-labeled product was resistent to digestion (FIG. 3B, lane 2). By contrast these conditions very efficiently digested the V-wt single-stranded RNA radioactively synthesized with T7 RNA polymerase (FIG. 3B, lanes 3 and 4). These nuclease S1 data confirmed that the opposite strand was indeed being synthesized in these reactions. The product of the reaction might be a double stranded RNA, but it could not be ruled out that the product was in fact single stranded and later annealed to the template RNA in the presence of high salt used in the nuclease reaction.

The RNA products were purified by electrophoresis on an 8% gel, excised, eluted from the gel, and then digested by ribonuclease T1. Products were analyzed by electrophoresis and compared to the patterns generated by RNase T1 digestion of internally labeled M-wt and V-wt control probes. As can be seen in FIG. 3C, the full

length RNA (lane 1) has the identical pattern as does the plus sense RNA, M-wt (lane 3), and it does not have the pattern of the V-wt RNA (lane 4). The observed patterns were essentially identical to that which is predicted from the sequence of the RNA and thus showed that the polymerase faithfully copied the V-wt template. The smaller RNA product, a doublet with most templates, was also digested with RNase T1. Its pattern was similar to that of the full length RNA product (FIG. 3C, lane 2) except the 14 base oligonucleotide was not present. Instead, a faint 13 base oligonucleotide was seen, thus mapping the termination of the short RNA to position 44, a site where two uridines would be incorporated. Since the amount of smaller RNA product decreased at higher UTP concentrations and disappeared when CTP was used as label, these bands appeared to be an artifact of low UTP concentrations in the polymerase reaction.

iii. CONDITIONS FOR THE POLYMERASE REACTIONS USING MODEL RNA TEMPLATES

It was found that protein samples containing about 30 ng of NP protein and about 200 pg total of the three P proteins would react optimally with 5 to 10 ng of RNA. By using cold competitor RNA, polyI-polyC, it was found that excess RNA nonspecifically inhibited transcription, possibly via non-specific binding of the NP protein (Kingsbury, et al., 1987, Virology 156: 396-403; Scholtissek and Becht, 1971, J. Gen. Virol. 10: 11-16). In the absence of nonspecific competitor, variations in the amount of template between 1 and 10 ng produced little change in the efficiency of RNA synthesis. The NP protein and RNA were present at about equal molar concentrations and these were each about a thousand-fold in excess of the moles of the complex (assuming it to be 1:1:1) formed by the three P proteins in the typical reaction.

Since these reconstituted RNPs were able to use ApG but not globin mRNA as primer, we tested these model RNPs for other variables of the transcription reaction. In all other ways tested, the reconstituted RNPs behaved in solution similarly to those RNPs purified from detergent disrupted virus. The optimum temperature for RNA synthesis was 30°C (FIG. 4A, lane 2) as has been repeatedly found for the viral polymerase (Bishop, et al., 1971, J. Virol. 8: 66-73; Takeuchi, et al., 1987, J. Biochem 101: 837-845; Ulmanen, et al., 1983, J. Virol. 45: 27-35). Also, the most active salt conditions were 60 mM NaCl (FIG. 4B, lane 2), again consistent with

conditions used by several groups (Bishop, et al., 1971, J. Virol. 8: 66-73; Honda, et al., 1988, J. Biochem. 104: 1021-1026; Shapiro, and Krug, 1988, J. Virol. 62: 2285-2290). Figure 4C shows a time-course experiment. The amount of RNA synthesis appeared to increase roughly linearly for the first 90 minutes, as was found for viral RNPs (Takeguchi, et al., 1987, J. Biochem. 101: 837-845).

iv. SPECIFICITY OF THE ELONGATION REACTION

Various RNAs were tested for suitability as templates for the RNA polymerase of influenza virus. The pV-wt plasmid clone was digested with either EcoRI, PstI or SmaI, and T7 polymerase was used to transcribe RNA. This resulted in RNAs identical to V-wt except for the addition of 5, 13 and 38 nt at the 3′ end. In FIG. 5A an overexposure of an autoradiograph is shown in order to demonstrate that no transcripts over background were observed in reactions which contained as template: two of the RNAs identical to V-wt except they contained 13 and 38 nt of extra sequence on the 3′ terminus (lanes 1 and 2); a single stranded DNA of identical sequence to that of V-wt (lane 4); and an unrelated 80 nt RNA generated by transcribing the polylinker of pIBI-31 with T3 RNA polymerase (lane 5). However, the V-Eco template, containing five extra nucleotides on the 3′ end, could be recognized and faithfully transcribed, although at approximately one-third the efficiency of the wild type V-wt RNA (FIG. 5B, lane 3). It is interesting to note that initiation on the V-Eco RNA by the influenza viral polymerase appeared to occur at the correct base since the transcribed RNA was the same size as the product from the V-wt template.

v. ANALYSIS OF THE PROMOTER REGION FOR THE VIRAL RNA POLYMERASE

The original construct used for these studies contained the sequences of both RNA termini of genomic RNAs which could base pair and thus form a panhandle. This was done since it was shown that the vRNA in virions and in RNPs in infected cells was in circular conformation via the 15 to 16 nt long panhandle (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). It was further shown that the viral polymerase was bound to the double stranded structure (Honda, et al., 1988, J. Biochem. 104: 1021-1026), thus leading to the suggestion that the promoter for RNA synthesis was the panhandle. In order to test

whether the panhandle was an absolute requirement for recognition, the following templates were used: the plasmid pV-wt was digested with DraI prior to transcription by the T7 polymerase (FIG. 2). This should result in an RNA molecule of 32 nt containing only virus-specific sequences from the 5′ end of the RNA. When this RNA was used as template, no apparent product was produced (FIG. 5B, lane 2). Therefore the 3′ terminus of virion RNA was required for this reaction. This finding was consistent with the fact that the initiation site at the 3′ end of V-wt was not present in V-Dra. A second plasmid clone was produced which deleted the 5′ terminal sequences but kept intact the 3′ terminus. This clone, pV-d5′, when digested with MboII and used for transcription by T7 polymerase produced a major transcript of 30 nt and minor species of 29 and 31 nt. Surprisingly, this template was recognized and copied by the influenza viral polymerase. FIG. 7, lane 1, shows that the product of the viral RNA polymerase reaction with V-d5′ contains multiple bands reflecting the input RNA. When the products shown in FIG. 7, lane 1, were eluted from gels and subjected to RNase T1 analysis, the pattern expected of the transcription product of V-d5′ was observed. Since the V-d5′ RNA template was copied, the panhandle was not required for viral polymerase binding and synthesis.

Although the 5′ terminus was not required for synthesis by the polymerase, a distinct possibility was that V-wt RNA might be a preferred template as compared to V-d5′. In order to examine this, reactions were done in which the templates were mixed. The V-wt RNA was present at 5 ng in each reaction. The V-d5′ was absent (FIG. 6, lane 1) or was present at a 1/5 molar ratio (FIG. 6, lane 2) or a 1/1 molar ratio (FIG. 6, lane 3). The relative intensities of the bands from each RNA were determined by densitometry of the autoradiograph. The values were corrected for the amount of the radioactive nucleotide, UTP, which could be incorporated into each product, and the value was normalized so that the level of synthesis in each lane was set equal to one. The level of copying of V-wt decreased as V-d5′ was increased. When V-d5′ was present in one fifth molar ratio, its corrected level of synthesis was about one fourth of that from V-wt (FIG. 6, lane 2). When the two templates were present in equimolar amounts, the level of synthesis from V-wt was about 60% of the total (FIG. 6, lane 3) which might be within the expected range of experimental error for equivalent levels of synthesis. Similar results were obtained when V-d5′ RNA was

kept constant and the V-wt RNA was varied. It was thus concluded that the panhandle-containing V-wt RNA was not greatly favored over the template RNA which only contained the proper 3′ terminus.

vi. THE VIRAL POLYMERASE DOES NOT COPY RNA TEMPLATES CONTAINING PLUS-SENSE TERMINI

As described earlier, the influenza RNA polymerase performs three distinct activities during the course of an infection. Two activities involve the transcription of genome sense RNA and the third involves copying of the complementary sense RNA into vRNA. We therefore constructed an RNA template which contained the 5′ and 3′ termini of the complementary sense RNA of segment 8 (M-wt; FIG. 2).

When the M-wt RNA was used as template, little synthesis was observed (FIG. 5B, lane 4). In two experiments used for quantitation, the average level of synthesis from M-wt RNA was 4% that of V-wt. In comparing the V-wt and M-wt RNA promoters, the M-wt has only three transition changes and one point insertion within the 3′ 15 nucleotides. These include a G to A change at position 3, a U to C change at position 5, a C to U change at position 8 and an inserted U between the ninth and tenth nucleotides (see Table II, below). In order to determine which of the four point differences in the 3′ termini were responsible for the specificity, many combinations of these were prepared and assayed for efficiency as a template (FIG. 7). These templates were derivatives of V-d5′ since they did not contain the 5′ terminus. The results of densitometry scans of several experiments are outlined in Table II.

TABLE II

QUANTITATIVE COMPARISON OF THE EFFECT OF POINT MUTATIONS IN THE PROMOTER SEQUENCE*

Template	3′ sequence	Level of RNA Synthesis
V-d5′	CACCCUGCUUUUGCU-OH	1
V-A3	CACCCUGCUUUUACU-OH	0.4
V-C5	CACCCUGCUUCUGCU-OH	1.0
V-d$U_{25}U_8$	CACCCUGUUUUUGCU-OH	1.0
V-U_8A_3	CACCCUGUUUUUACU-OH	0.08
V-U_8C_5	CACCCUGUUUCUGCU-OH	0.3
V-iU_{10}	CACCCUUGCUUUUGCU-OH	0.7
V-i$U_{10}A_3$	CACCCUUGCUUUUACU-OH	0.06
V-i$U_{10}U_8A_3$	CACCCUUGUUUUUACU-OH	0.2
V-i$U_{10}U_8C_5A_3$	CACCCUUGUUUCUACU-OH	0.2

* Sequences of V-wt, M-wt and V-d5′ are shown in FIG. 2. All other RNAs are identical to V-d5′ except for the indicated positions. The subscripted number indicates the distance from the 3′ end of a change, and d and i refer to deleted or inserted nucleotides.

As shown in Table II, single point changes in V-d5′ were equally well copied as compared to V-d5′ itself, except for the V-A_3 RNA which was copied at 40% efficiency (FIG. 7, lane 10; Table II). When RNAs with two changes were tested, the activity generally dropped to very low levels (FIG. 7, lanes 3, 4, and 5). Therefore, these experiments confirmed that the specificity of the reactions for V-wt over M-wt was the result of the combination of the nucleotide changes present at the 3′ terminus of M-wt.

vii. CAP-ENDONUCLEASE PRIMED RNA SYNTHESIS

The method of purifying the viral polymerase was modified in order to decrease loss of protein during dialysis. Rather than using the Amicon centricon-*10* dialysis system, the enzyme was dialyzed in standard membranes resulting in higher concentrations of all four viral core proteins. The pattern of the protein gel of this preparation was identical to that shown in FIG. 1, lane 4, except that there is no BSA-derived band. It was found that 5 μl of this preparation, containing 150 ng of NP and 5 ng total of the three polymerase proteins, reacted optimally with 10 to 40 ng of model RNA template. However, the use of higher levels of protein increased the background, possibly due to higher levels of contaminating RNAs (virion RNAs not removed by CsCl centrifugation) yielding products of the size class around 50-75 nt, complicating analysis of RNA templates containing a length of 50 nt.

This high concentration polymerase preparation was now active in cap-endonuclease primed RNA synthesis (FIG. 8A, lane 4) and also in primer-independent replication of the template RNA (FIG. 8A, lane 2). When globin mRNA was used as primer for transcription from the 30 nt V-d5′ template, a triplet of bands of size about 42 to 44 nt was apparent as product (FIG. 8A, lane 4), consistent with cleavage of the cap structure at about 12 nt from the 5′ end of the mRNA and use of this oligonucleotide to initiate synthesis from the 30 nt model template. Since excess RNA inhibits RNA synthesis, probably via nonspecific binding of NP in vitro as discussed above, the optimal amount of cap donor RNA added to each reaction was found to be 100 ng, which is much lower than is usually used with preformed RNP structures (e.g., Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952-3956). The most effective primer was ApG (FIG. 8A, lane 5 and lighter exposure in lane 6). The product migrates slower than that of the input template (FIG. 8A, lane 1) or the product in the

absence of primer (FIG. 8A, lane 2) probably since the 5′ terminus of the ApG product is unphosphorylated. The intensity of the ApG-primed product was about ten-fold higher than that of the cap-primed product, but at 0.4 mM, ApG was at a 60,000-fold molar excess of the concentration of the cap donors. Thus, although the intensity of the product band from cap-priming was about ten-fold lower than that from ApG priming, the cap-primed reaction was about 6000-fold more efficient on a molar basis. This value is similar to the approximately 4000-fold excess efficiency observed previously for the viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952-3956). It has been previously shown that cap donor RNAs containing a cap 0 structure, as in BMV RNA, are about ten-fold less active in priming the influenza viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952-3956). This unusual cap specificity was shared by the reconstituted RNPs studied here as the specific product from the model RNA was greatly decreased in reactions containing BMV RNA as cap donor. A 30 nt product was observed in lanes 2-4, probably due to primerless replication of the model template.

That the product RNAs were of the opposite sense of the input template V-d5′ was shown by nuclease S1 analysis (FIG. 8B). The ApG-primed (FIG. 8B, lanes 1 and 2) and the primerless (FIG. 8B, lanes 3 and 4) RNA products were essentially nuclease resistent. The product of the cap-primed reaction (FIG. 8B, lanes 5 and 6) was partially sensitive to nuclease as about 12 nt were digested from the product. These results were most consistent with the 5′ 12 nt being of mRNA origin as has been shown many times for influenza virus-specific mRNA synthesis.

The promoter specificity of this polymerase preparation in reactions primed with ApG was found to be essentially identical to those for the lower concentration enzyme as shown earlier. However, attempts thus far to perform similar analyses of promoter specificity with the primerless and cap-primed reactions have been frustrated by the comparatively high levels of background, thus making quantitation difficult.

viii. REPLICATION OF GENOMIC LENGTH RNA TEMPLATES

A full-length 890 nt RNA identical to the sequence of A/WSN/33 segment 8 was prepared by T7 RNA polymerase transcription of plasmid DNA, pHgaNS, which had been digested with restriction endonuclease HgaI. This RNA was copied in ApG-primed reactions containing 10 μl of the high concentration polymerase (FIG. 9, lane

8). That the RNA was in fact a copy of the template was demonstrated by its resistance to nuclease S1 (FIG. 9, lane 9). A similar product was observed in the absence of primer (FIG. 9, lanes 2 and 3). Confirmation that these product RNAs were full length copies of the template was done by RNase T1 analysis. Virion RNA purified from phenol-extracted A/PR/8/34 virus was similarly copied in ApG primed reaction (FIG. 9, lanes 10 and 11) and in the absence of primer (FIG. 9, lanes 4 and 5). Interestingly, the product from replication of the HA gene was at greatly reduced levels. The 3′ end of this RNA differs from that of segment 8 only at nucleotides 14 and 15, suggesting importance for these nucleotides in the promoter for RNA synthesis. In addition, we found that when whole viral RNA was used in the reconstituted RNPs, the level of acid precipitable counts was about 70% of that observed with native RNPs. The viral polymerase was also able to copy these full length RNAs when globin mRNA was used in cap-primed reaction.

6. EXAMPLE: EXPRESSION AND PACKAGING OF A FOREIGN GENE BY RECOMBINANT INFLUENZA VIRUS

The expression of the chloramphenicol transferase gene (CAT) using rRNPs is described. The rRNPs were prepared using pIVACAT (originally referred to as pCATcNS), a recombinant plasmid containing the CAT gene. The pIVACAT plasmid is a pUC19 plasmid containing in sequence: the T7-promoter; the 5′- (viral-sense) noncoding flanking sequence of the influenza A/PR8/34 RNA segment 8 (encodes the NS proteins); a BglII cloning site; the complete coding sequence of the chloramphenicol transferase (CAT) gene in the reversed and complemented order; the 3′- (viral-sense) noncoding NS RNA sequence; and several restriction sites allowing run-off transcription of the template. The pIVACAT can be transcribed using T7 polymerase to create an RNA with influenza A viral-sense flanking sequences around a CAT gene in reversed orientation.

The in vivo experiments described in the subsections below utilized the recombinant RNA molecule described containing sequences corresponding to the untranslated 3′ and 5′ terminal sequences of the NS RNA of influenza virus A/PR/8/34 flanking the antisense-oriented open reading frame of the CAT gene. This RNA was mixed with purified influenza virus polymerase complex and transfected into MDCK (or 293) cells. Following infection with influenza A/WSN/33 virus, CAT activity was

measured in the RNP-transfected cells and amplification of the gene was indicated. In addition, the recombinant influenza virus gene was packaged into virus particles, since CAT activity was demonstrated in cells following infection with the recombinant virus preparation.

a. MATERIALS AND METHODS

In order to get the flanking sequences of the NS RNA fused to the coding sequence of the CAT gene, the following strategy was used. Two suitable internal restriction sites were selected, close to the start and stop codon of the CAT gene, that would allow the replacement of the sequences flanking the CAT gene in the pCM7 plasmid with the 3′- and 5′- NS RNA sequences. At the 5′ end, a SfaNI site was chosen (which generates a cut 57 nt from the ATG), and at the 3′- end a ScaI site which generates a cut 28 nt from the end of the gene (stop codon included). Next, four synthetic oligonucleotides were made using an Applied Biosystems DNA synthesizer, to generate two double-stranded DNA fragments with correct overhangs for cloning. Around the start codon these oligonucleotides formed a piece of DNA containing a XbaI overhand followed by a HgaI site and a PstI site, the 3′- (viral-sense) NS sequence immediately followed by the CAT sequence from start codon up to the SfaNI overhang (underscored). In addition a silent mutation was incorporated to generate an AccI site closer to the start codon to permit future modifications.

Xba I
Hga I Pst I Acc
5'-ctagacgccctgcagcaaaagcagggtgacaaagacataatggagaaaaaaatcac
3'tgcgggacgtcgttttcgtcccactgtttctgtattacctctttttttagtg

I SfaN I
tgggtataccaccgttgatatatcccaatcgcatcgtaaa- 3' oligo2
acccatatggtggcaactatatagggttagcgtagcatttcttg- 5' oligo1

Around the stop codon the two other oligonucleotides generated a piece of DNA as follows: a blunt-ended SCAI site, the CAT sequence from this site up to and including the stop codon (underlined) followed by a BglII site and a Xba I overhang.

Sca I Bgl II
5'-actgcgatgagtggcagggcggggcgtaatagat- 3' oligo3
3'-tgacgctactcaccgtcccgccccgcattatctagatc- 5' oligo4
XbaI

Using a single internal EcoRI site in the CAT sequence, the SfaNI/EcoRI and the EcoRI/ScaI fragment from pCM7 were independently cut out and purified from acrylamide gels. The SfaNI/EcoRI fragment was subsequently ligated with the synthetic DNA fragment obtained by annealing oligonucleotides 1 and 2 into a pUC19 plasmid that was cut with XbaI and EcoRI. The EcoRI/ScaI fragment was similarly cloned into an XbaI and EcoRI -digested pUC19 plasmid using oligonucleotides 3 and 4. The ligated DNA was transformed into competent DH5a bacteria, amplified, isolated and screened by means of restriction analysis using standard techniques.

The recombinants with the SfaNI containing insert were cut with XbaI and EcoRI and the plasmids with the ScaI insert were cut with EcoRI and BglII. The fragments were purified from acrylamide gel and cloned together into the pPHV vector which had been cut with XbaI and BglII. After transformation, white colonies were grown, analyzed by endonuclease digestion and selected clones were sequenced. The final clone, pCATcNS2, was grown in large amounts and sequenced from the flanking pUC sequences up to 300 nt into the CAT gene, revealing no discrepancies with the intended sequence, with the exception of a G to A transition in the CAT gene, which appeared silent.

i. VIRUSES AND CELLS

Influenza A/PR/8/34 and A/WSN/33 viruses were grown in embryonated eggs and MDCK cells, respectively (Ritchey et al. 1976, J. Virol. 18: 736-744; Sugiura et al., 1972, J. Virol. 10: 639-647). RNP-transfections were performed on human 293 cells (Graham et al., 1977, J. Gen. Virol. 36: 59-72) and on Madin-Darby canine kidney (MDCK) cells (Sugiura et al., 1972, supra).

ii. CONSTRUCTION OF PLASMIDS

Plasmid pIVACAT1, derived from pUC19, contains the coding region of the chloramphenicol acetyltransferase (CAT) gene flanked by the noncoding sequences of the influenza A/PR/8/34 RNA segment 8. This construct is placed under the control of the T7 polymerase promoter in such a way that the RNA transcript IVACAT1 contains in 5′ to 3′ order: 22 nucleotides derived from the 5′ terminus of the influenza virus NS RNA, an 8 nt linker sequence including a BglII restriction site, the CAT gene in negative polarity, and 26 nt derived form the 3′ end of the influenza virus NS RNA (FIG. 11).

pIVACAT1 was constructed in the following way: In order to obtain the correct 5′- end in pIVACAT1, the EcoRI-ScaI fragment of the CAT gene derived from plasmid pCM7 (Pharmacia) was ligated to a DNA fragment formed by two synthetic oligonucleotides. The sequence of these oligonucleotides are: 5′-ACTGCGATGAGTGGCAGGGCGGGGCGTAATA-GAT- 3′ (top strand), and 5′-CTAGATCTATTACGCCCCGCCCTGCCAC-TCATCGCAGT- 3′ (bottom strand). For the 3′- end of the insert in pIVACAT1 the SfaN 1-EcoRI fragment of the CAT gene was ligated to a DNA fragment made up of the synthetic oligonucleotides: 5′-C T A G A C G C C C T G C A G C A A A A G C A G G G T G A C -AAAGACATAATGGAGAAAAAAAATCACTGGGTATACCACCGTTGATATAT CCCAATCG-CATCGTAAA- 3′ (top strand), and 5′-GTTCTTTACGATGCGATTGGGAT-TTTGCTGCAGGGCGT- 3′ (bottom strand). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. These 5′ and 3′ constructs were ligated into pUC19 shuttle vectors digested with XbaI and EcoRI, grown up, cut out with EcoRI/BglII (5′ region) and XbaI/EcoRI (3′ region) and ligated into BglII/XbaI cut pPHV. The latter plasmid is similar to pV-WT described in Section 6, supra, except that it contains a BglII site which separates the noncoding

terminal sequences of the influenza A virus NS RNA segment. The final clone pIVACAT1 (FIG. 1) was grown up and the DNA was partially sequenced starting from the flanking pUC sequences and reaching into the CAT gene. No changes were found as compared to the expected sequences with the exception of a silent G to A transition in the CAT gene at position 106 relative to the start of the IVACAT1 RNA.

iii. T7 RNA TRANSCRIPTION

Plasmid pIVACAT1 was digested with HgaI (FIG. II), to allow run-off transcription. The 5 nt overhang generated by this enzyme was filled in with Klenow enzyme (BRL) and the DNA was purified over a spin column (Boehringer). The T7 polymerase reaction was performed using standard procedures in the presence of Rnasin (Promega). Template DNA was removed from Rnase free Dnase I (Promega). The RNA was purified over Qiagen tip-5 columns (Qiagen, Inc.) and quantitated using 4% polyacrylamide gels which were silver stained. NS RNA was prepared from plasmid pHgaNS in the same way.

iv. PURIFICATION OF INFLUENZA A VIRUS POLYMERASE AND IN VITRO TRANSCRIPTION

The RNA polymerase complex was purified from influenza A/PR/8/34 as described in Section 6, supra. In vitro transcriptions of cold IVACAT1 or HgaNS RNA template were carried out using the conditions which have been described in Section 6, supra. Radiolabeled transcripts were analyzed on 4% acrylamide gels.

v. RNP-TRANSFECTION OF MDCK AND 293 CELLS

35 mm dishes containing approximately 10^6 cells were treated with 1 ml of a solution of 300 μg/ml DEAE-dextrin, 0.5% DMSO in PBS/gelatine (0.1 mg/ml gelatine) for 30 minutes at room temperature. After removal of this solution, 200 μg of μl PBS/gelatine containing 1 μg IVACAT1 RNA (1-2 μl), 20 μl of the purified polymerase preparation and 4 μl of Rnasin was added to the cells and incubated for 1 hour at 37° C. This was followed by the addition of gradient purified influenza A/WSN/33 virus (moi 2-10). After incubation for one hour at 37° C, 2.5 ml of either DMEM + 10% FCS media (293 cells) or MEM media (MDCK cells) was added. In some experiments MDCK cells were first infected and subsequently RNP-transfected. Harvesting of cells was done in NET buffer or in media, using a rubber policemen (MDCK cells), or by gentle suspension (293 cells). Cells were spun down and the

pellets were resuspended in 100 μl of 0.25 M Tris buffer, pH 7.5. The samples were subsequently freeze-thawed three-times and the cell debris was pelleted. The supernatant was used for CAT assays.

vi. PASSAGING OF VIRUS FROM RNP-TRANSFECTED CELLS

MDCK cells were infected with helper virus and RNP-transfected 2 hours later as described above. After 1 hour cells and media were collected and cells were spun down. 100 μl of the supernatant media, containing virus, was added to 35 mm dishes with MDCK cells. After 12 hours these cells and media were collected and assayed for CAT activity. Virus contained in this supernatant media was used for subsequent rounds of infection of MDCK cells in 35 mm dishes.

vii. CAT ASSAYS

CAT assays were done according to standard procedures, adapted from Gorman et al., 1982, Mol. Cell. Biol. 2: 1044-1051. The assays contained 10 μl of ^{14}C chloramphenicol (0.5 μCi; 8.3 nM; NEN), 20 μl of 40 mM acetyl CoA (Boehringer) and 50 μl of cell extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were 16-18 hours.

b. RESULTS

rRNA templates were prepared from HgaI digested, end filled linearized pCATcNS using the bacteriophage T7 RNA polymerase as described in Section 6. The rRNA templates were combined with the viral RNA polymerase complex prepared as described in Section 6.1.1., and the resulting rRNPs were used to transfect MDCK and 293 cells lines which were superinfected with influenza A/WSN33. In each cell line transfected with the rRNPs, high levels of expression of CAT was obtained 6 hours post-infection. In addition, virus stocks obtained 24 hours post-infection synthesized high levels of CAT enzyme after subsequent passage in MDCK cells. The CAT-RNP was packaged into virus particles.

i. SYNTHESIS OF IVACAT1 TEMPLATE RNA

In order to study the transcription and replication signals of influenza A virus RNAs in vivo, we constructed plasmid pIVACAT1 (FIG. II) which directs the synthesis of an NS RNA-like transcript. This RNA shares the 22 5′ terminal and the 26 3′ terminal nucleotides with the NS RNA of influenza A/PR/8/34 virus and contain -- instead of the coding sequences for the NS1 and NS2 proteins -- those for a full-

length CAT protein. For cloning purposes it also contains eight additional nucleotides including a BglII site between the stop codon of the CAT gene and the stretch of U's in the 5′ noncoding region. The T7 promoter adjacent to the 5′ noncoding sequences and the HgaI site downstream of the 3′ end allow for the exact tailoring of the 5′ and 3′ ends. Run-off transcription using T7 polymerase generates a 716 nt long RNA: Fig. 12, lanes 2-4 show that this RNA is of discrete length and shorter than the 890 nt long marker NS RNA, which was synthesized by T7 transcription of pHgaNS (lane 1).

ii. THE IVACAT1 RNA IS TRANSCRIBED IN VITRO BY THE INFLUENZA A VIRUS RNA POLYMERASE

In the examples described in Section 6, it was demonstrated that synthetic RNAs containing at the 3′ end the 15 3′ terminal nucleotides of influenza virus RNA segment 8 can be transcribed in vitro using purified influenza A virus RNA polymerase. We tested whether unlabeled IVACAT1 RNA could be transcribed in a similar way. FIG. 12 lane 5 shows that the in vitro transcription reaction generated an RNA of discrete length and similar size to the product of the T7 transcription reaction suggesting synthesis of a full length product.

iii. RNP-TRANSFECTION AND CAT ACTIVITY

Since the recombinant CAT RNA could be transcribed in vitro, a system was designed to test whether this RNA can be recognized and replicated in vivo (FIG. 13). Recombinant RNA was mixed with the purified polymerase to allow formation of viral RNP-like particles. To facilitate the association, the RNA/polymerase mixture was incubated in transcription buffer without nucleotides for 30 minutes at 30°C prior to RNP-transfection. In some experiments, this preincubation step was omitted. RNP-transfections were either preceded or followed by infection with influenza A/WSN/33 virus, since the production of viral polymerase protein was expected to be necessary for efficient amplification of the gene. The cells used were either MDCK cells, which are readily susceptible to influenza A/WSN/33 virus infection, or human 293 cells, which support infection at a slower rate.

In order to determine whether the minus sense IVACAT1 RNA could be amplified and transcribed in vivo, an experiment was performed in 293 cells. Cells were transfected with RNP, virus infected one hour later and harvested at various times

post-infection. FIG. 14A shows that at early times post infection only background levels of CAT activity were detected (lanes 5, 7 and 9). However, significant levels of CAT activity appeared seven hours after virus infection (lane 11). A similar level of CAT activity was detected two hours later (lane 13). There were background levels of CAT activity in the mock transfected cells at any time point (lanes 6, 8, 10, 12 and 14), and in control cells not infected with A/WSN/33 virus (lanes 1-4).

Preincubation of RNA and polymerase complex was not necessary for successful RNP-transfection. As can be seen in FIG. 14B, lanes 2 and 3, preincubation might actually cause a decrease in CAT activity, presumably due to RNA degradation during preincubation. In another control experiment, infection by helper virus of RNP-transfected cells was omitted (FIG. 14B, lanes 4 and 5). Since these lanes show no CAT activity we conclude that the IVACAT1 RNA is amplified specifically by the protein machinery supplied by the helper virus. In an additional control experiment, naked RNA was transfected into cells which were subsequently helper-infected or mock-infected. Again, no CAT activity was detected in these samples (FIG 14B, lanes 6-9). Finally virus-infected cells which were not transfected with recombinate CAT-RNP also did not exhibit endogenous acetylation activity (FIG. 14B, lane 10). It thus appears that addition of the purified polymerase to the recombinant RNA as well as infection of cells by helper virus is important for successful expression of the CAT enzyme.

Experiments were also performed using MDCK cells, the usual tissue culture host cell for influenza virus (FIG. 14C). When the reconstituted recombinant CAT-RNP complex was transfected 1 hour before virus infection, little CAT activity was observed at 7 hours post virus infection (FIG. 14C, lane 1). When RNP-transfection was accomplished 2 hours after virus infection, expression of CAT was greatly enhanced at 7 hours post-virus infection (FIG. 14C, lane 3). Therefore, MDCK cells are also viable host cells for these experiments.

iv. THE CAT-RNP IS PACKAGED INTO VIRUS PARTICLES

Since the recombinant CAT RNA can be replicated in vivo via helper virus functions, we examined whether virus produced in RNP-transfected and helper virus infected cells contained the CAT gene. MDCK cells were used in the experiment because they yield higher titers of infectious virus than 293 cells. MDCK cells were

infected with A/WSN/33 virus, RNP-transfected 2 hours later and allowed to incubate overnight. At 14 hours post infection, media was harvested and cells were pelleted. Virus supernatant was then used to infect new MDCK cell monolayers. The inoculum was removed after 1 hour and cells were harvested at 12 hours post infection and assayed for CAT activity. FIG. 15 reveals that the virus preparation induces a level of CAT activity (lanes 2 and 3) which is significantly above control (lane 1). In this case, the addition of helper virus to the inoculum did not increase CAT activity (lane 4). Further passaging of supernatant virus on fresh MDCK cells did not result in measurable induction of CAT activity. This is not surprising as there is no selective pressure for retaining the CAT gene in these viral preparations. We excluded the possibility that we were transferring the original RNA/polymerase complex by pretreating the inocula with RNase. This treatment destroys viral RNPs of influenza virus (Pons et al., 1969, Virology 39: 250-259; Scholtissek and Becht, 1971 J. Gen. Virol. 10: 11-16).

7. RESCUE OF INFECTIOUS INFLUENZA VIRUSES USING RNA DERIVED FROM SPECIFIC RECOMBINANT DNAs

The experiments described in the subsections below demonstrate the rescue of infectious influenza viruses using RNA which is derived from specific recombinant DNAs. RNAs corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN virus) were transcribed in vitro from appropriate plasmid DNAs and -- following the addition of purified influenza virus polymerase complex (as described in Section 6.1.1. supra) -- were transfected into MDBK cells as described in Section 7, supra. Superinfection with helper virus, lacking the WSN NA gene, resulted in the release of viruses containing the WSN NA gene. Thus, this technology allows the engineering of infectious influenza viruses using cDNA clones and site-specific mutagenesis of their genomes. Furthermore, this technology may allow for the construction of infectious chimeric influenza viruses which can be used as efficient vectors for gene expression in tissue culture, animals or man.

The experiments described in Sections 6 and 7 supra, demonstrate that the 15 3′ terminal nucleotides of negative strand influenza virus RNAs are sufficient to allow transaction in vitro using purified influenza virus polymerase proteins. In addition, the studies using the reporter gene chloramphenicol acetyltransferase (CAT) show that the

22 5′ terminal and the 26 3′ terminal nucleotides of the viral RNAs contain all the signals necessary for transcription, replication and packaging of influenza virus RNAs. As an extension of these results, a plasmid, pT3NAv, was constructed which contained the complete NA gene of influenza A/WSN/33 virus downstream of a truncated T3 promoter (FIG. 16). Therefore, runoff transcription of this plasmid, cut at the Ksp632I site, yields an RNA which is identical to the true genomic NA gene of the WSN virus (Fig. 17, lane 3). This RNA was then incubated with purified polymerase (purified as described in Section 6.1.1) and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus which did not contain the WSN virus NA. The choice of WSN-HK helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, it was shown that the WSN-HK virus can only form plaques in MDBK cells when protease is added to the medium. This is in marked contrast to WSN virus (isogenic to WSN-HK virus except for the neuraminidase gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (Schulman et al., 1977, J. Virol. 24: 170-176).

a. MATERIALS AND METHODS

i. VIRUSES AND CELLS

Influenza A/WSN/33 virus and A/WSN-HK virus were grown in Madin-Darby canine kidney (MDCK) cells and embryonated eggs, respectively (Sugiura et al., 1972, J. Virol. 10: 639-647; Schulman et al., 1977, J. Virol. 24: 170-176). Influenza A/PR/8/34 virus was also grown in embryonated eggs. Madin-Darby bovine kidney (MDBK) cells were used for the transfection experiments and for selection of rescued virus (Sugiura et al., 1972, J. Virol. 10: 639-647).

ii. CONSTRUCTION OF PLASMIDS

The pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 plasmids were constructed by PCR-directed mutagenesis using a cloned copy of the WSN NA gene, which was obtained following standard procedures (Buonagurio et al., 1986, Science 232: 980-982). To construct pT3NAv, the following primers were used: 5′-CGGAATTCTCTTCGAGCGAAAGCAGGAGTT-3′ and 5′-CCAAGCTTATTAACCCTCACTAAAAGTAGAAACAAGGAGTTT-3′. After 35 cycles in a thermal cycler (Coy Lab Products, MI), the PCR product was digested with

EcoRI and HindIII and cloned into pUC19. Plasmid pT3NAv mut 1 was constructed in a similar fashion except that the sequence of the primer was altered (FIG. 16). Plasmid pT3NAv mut 2 was constructed by cassette mutagenesis through the digestion of pT3NAv with PstI and NcoI and relegation in the presence of the synthetic oligonucleotides - 5'-CATGGGTGAGTTTCGACCAAAATCTAGATTATAAAATAGGATACATATG CA-3' and 5'-AATGTATCCTATTTTATAATCTAGATTTTGGTCGAAACTCACC-3'. Oligonucleotides were synthesized on an applied Biosystems DNA synthesizer. The final clones pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 were grown up and the DNAs were partially sequenced starting from the flanking pUC19 sequences and reaching into the coding sequences of the NA gene. The mutations in pT3NAv mut 2 were also confirmed by sequencing.

iii. PURIFICATION OF INFLUENZA A VIRUS POLYMERASE AND RNP TRANSFECTION IN MDBK CELLS

The RNA polymerase complex was purified from influenza A/PR/8/34 virus as described in Section 6.1.1, supra, and was then used for RNP transfection in MDBK cells using the protocol described in Section 7, supra, except that WSN-HK virus was used as helper virus at an moi of 1. RNAs used for RNP transfection were obtained by phenol extraction of purified virus or by transcription (using T3 polymerase) of pT3NAv, pT3NAv mut 1 and pT3NAv mut 2. All plasmids were digested with Ksp632I, end-filled by Klenow enzyme (BRL) and then transcribed in a runoff reaction a described in Section 7, supra.

b. RESULTS

i. RESCUE OF INFECTIOUS INFLUENZA VIRUS IN MDBK CELLS USING RNA DERIVED FROM RECOMBINANT PLASMID DNA

A plasmid, pT3NAv, was constructed to contain the complete NA gene of influenza WSN virus downstream of a truncated T3 promoter (FIG. 16). Runoff transcription of the plasmid, cut at the Ksp632I site, yields an RNA which is identical in length to the true genomic NA gene of the WSN virus (FIG. 17, lane 3). This RNA was then incubated with purified polymerase and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus. The

choice of WSN-HK virus as helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, its was shown that the WSN-HN virus can only form plaques in MDBK cells when protease is added to the medium (Schulman et al., 1977, J. Virol. 24: 170-176). This is in marked contrast to WSN virus (isogenic to WSN-HK helper virus except for the neuraminidase gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (Sugiura et al., 1972, J. Virol. 10: 639-647). MDBK cells were first infected with the WSN-HK helper virus and RNP-transfected one hour after virus infection. Following overnight incubation in the presence of 20μg/ml plasminogen, supernatant from these cells was then amplified and plaqued in MDBK cells in the absence of protease in the medium. The appearance of plaques in MDBK cells (Schulman et al., 197, J. Virol. 10: 639-647) indicated the presence of virus which contained the WSN virus NA gene, since supernatant from control experiments of cells infected only with the WSN-HK virus did not produce plaques. In a typical experiment involving the use of a 35 mm dish for the RNP-transfection, 2.5 x 10^2 plaques were observed.

In another control experiment, synthetic NA RNA was used which was derived from plasmid pT3NAv mut 1 (FIG. 16). This RNA differs from the wild type NA RNA derived from pT3NAv by a single nucleotide deletion in the nontranslated region of the 5′ end (FIG. 16). RNP-transfection of MDBK cells with this RNA and superinfection with WSN-HK virus did not result in the formation of rescued virus. This negative result is readily explained since we have shown in Section 6 and 7, supra, that the essential sequences for the recognition of viral RNA by viral polymerases as well as the packaging signals are located within the 3′ and 5′ terminal sequences of the viral RNAs. However, we cannot exclude the possibility that rescue of virus using this mutated RNA does occur, albeit at an undetected frequency.

ii. RNA ANALYSIS OF RESCUED VIRUS

Virus obtained in the rescue experiment was plaque purified, amplified in MDBK cells and RNA was extracted from this preparation. The RNA was then analyzed by electrophoresis on a polyacrylamide gel. FIG. 17 shows the RNA of the helper virus WSN-HK (lane 1) and the synthetic NA RNA (lane 3), which was transcribe by T3 polymerase from plasmid pT3NAv. The migration pattern of the RNAs of the rescued virus (lane 2) is identical to that of control WSN virus (lane 4).

Also, the NA RNAs in lanes 2 and 4 migrate at the same position as the NA RNA derived from cDNA (lane 3) and faster than the HK virus NA band in the helper WSN-HK virus (lane 1). These experiments support the conclusion that as a result of the RNP-transfection, infectious virus was formed containing WSN virus NA RNA derived from cDNA.

iii. RESCUE OF INFECTIOUS INFLUENZA VIRUS USING VIRION RNA

In another transfection experiment, RNA extracted from purified WSN virus was employed. When this naked RNA is transfected together with the polymerase proteins into helper virus infected cells, rescue of WSN virus capable of replicating in MDBK cells is observed. RNA isolated from an amplified plaque in this experiment is analyzed in lane 5 of FIG. 17 and shows a pattern indistinguishable from that of the control of WSN virus in lane 4.

iv. INTRODUCTION OF SITE-SPECIFIC MUTATIONS INTO THE VIRAL GENOME

The experiments described so far involved the rescue of influenza WSN virus. Since the synthetic RNA used in these experiments is identical to the authentic WSN NA gene, the unlikely possibility of contamination by wild type WSN virus could not be rigorously ruled out. Therefore, we introduced five silent point mutations in the coding region of the NA gene in plasmid pT3NAv. These mutations were introduced by cassette mutagenesis through replacement of the short NcoI/PstI fragment present in the NA gene. The five mutations in the cDNA included a C to T change at position 901 and a C to A change at position 925, creating a new XbaI site and destroying the original PstI site, respectively. In addition, the entire serine codon at position 887-889 of the cDNA clone was replaced with an alternate serine triplet (FIG. 17). RNP-transfection of this mutagenized RNA (pT3NAv mut 2) and helper virus infection of MDBK cells again resulted in the rescue of a WSN-like virus which grew in MDBK cells in the absence of added protease. When the RNA of this virus was examined by sequence analysis, all five point mutations present in the plasmid DNA (FIG. 16) were observed in the viral RNA (FIG. 18). Since it is extremely unlikely that these mutations evolved in the wild type influenza WSN virus, we conclude that successful rescue of infectious influenza virus containing five site-specific mutations was achieved via RNP-transfection of engineered RNA.

9. ELECTROPORATION OF RNPs AND NP AND M GENE RESCUE

As an alternative method to the DEAE-dextran transfection protocol set forth in Section 7.1.5., electroporation can be used to transfer RNPs into cells. Electroporation has been used for transfecting DNA or RNA alone into cells. See for example, Liljestrom, et al, (1991), J. Virol. 65:4107-13. However, its utility in transfecting other molecules or substances, and specifically in transfecting reconstituted negative strand virus RNP, has to our knowledge not been explored, presumably due to doubts as to the ability of the technique to work with molecules and/or substances that exist in complexes. Due to the high background level of helper virus required and the inherent, relatively low transfection efficiency of the DEAE-dextran transfection method, that method is most effective when a highly stringent selection system exists for the transfectant virus, which has the ability of preventing or eliminating "break through" of the helper virus. It has been discovered that electroporation of RNPs not only works, but is more efficient that DEAE-dextran in delivering negative strand viral RNPs inside cells. Because it was believed that electroporation might dissociate the protein-RNA complex comprising the reconstituted negative strand virus RNP, these results are surprising and unexpected.

The effectiveness of electroporation for the generation of recombinant negative strand viruses is demonstrated in this section in two sets of rescue experiments using temperature-sensitive (ts) influenza virus as helper. In the first set of experiments, the NP gene, which is about 1565 nt in length, was rescued. First, a plasmid, designated as pT3WSN-NPmt, was constructed that contains the complete NP gene encoding the nucleoprotein of influenza A/WSN/33 virus and a truncated bacteriophage T3 promoter. A silent mutation was also introduced into the NP gene at position 282 (adenosine replaced with guanosine), which destroyed the unique XbaI site in the cDNA copy of the NP gene, to enable detection of the heterologous rescued NP sequence in viral progeny. The influenza ts56 virus, Sugiura, et al., 1972, J. Virology 10: 639-47, which has a defect in the NP gene that prevents virus growth at temperatures over 39° C, was used as helper. The influenza RNP complexes were prepared as described in Section 6 and the rescue was done as described in Example 7, except that one group of RNPs were transfected using the electroporation technique rather than the DEAE-dextran protocol described earlier. From 36 viruses isolated

after electroporation, 5 were successful transfectants. In contrast, none of the 36 viruses isolated after DEAE-dextran transfection were successful transfectants.

In Section 9.2, in the second set of experiments, the M gene of influenza A/WSN/33 virus, 1027 nucleotide in length, was rescued. Using standard reverse transcription polymerase chain reaction techniques (RT-PCR) the entire M gene was cloned into the pUC19 cloning vector. The resultant plasmid, pT3WSN-Mmt, contained a truncated T3 bacteriophage RNA polymerase promoter, which allows the synthesis of M RNA *in vitro* by T3 RNA polymerase using Ksp632I linealized plasmid as template. In addition, two silent mutations were introduced into the M gene at positions 286 (cytidine replaced with uridine) and 289 (adenosine replaced with guanosine) (using the convention of numbering from the N-terminal nucleotide). Both mutations were designed as markers for the transfected M gene. The mutation at position 289 of adenosine to guanosine created a unique BamHI restriction enzyme site in the M gene. RNA derived from plasmid pT3WSN-Mmt was then mixed with purified influenza virus NP and polymerase proteins to form M RNP *in vitro*. This reconstituted M RNP was transfected using electroporation into Influenza ts51 infected cells. The Influenza ts51 virus, see Rey, et al, 1992, J. Virol. 66:5815-24, has defect on its M gene that prevents virus growth at temperatures over 39° C. After the transfection, the supernatant was plaqued in Mardin-Darby kidney (MDCK) cells at the non-permissive temperature, ie., 39°C. Individual plaques were isolated and the origin of the M gene was analyzed by RT-PCR and restriction enzyme digestion. Both introduced mutations were confirmed by sequencing of the PCR product derived from viral RNA.

These experiments demonstrate the utility of using electroporation to transfect the host cells with recombinant RNP. Effecting the transfection by electroporation results in more efficient delivery of the negative strand viral RNPs inside the host cells. While the method is demonstrated using influenza, this demonstration is intended to be exemplary. The method as disclosed herein will work with any negative strand virus RNP complex. Additionally, although the method is demonstrated using the NP, NA and M genes of influenza, any of the other genes of influenza may be used in the electroporation method disclosed herein.

Another aspect of this invention involves the mutagenesis of selected segments of the influenza viral genome, for example the segments encoding the M gene and the NP gene. The M gene of influenza virus encodes at least two known matrix proteins, M1 and M2. See Lamb, et al., 1980, Cell 21: 475-85. The M1 protein lines the inner surface of the viral envelope. The M2 protein has ion-channel activity. Pinto, et al., 1992, Cell 69: 517-28. The M and NP genes are both involved in viral replication. See the discussion at Section 2.1, infra. Several studies have shown that M and NP genes are associated with high-growing properties of influenza virus in embryonated eggs. See Baez, et al., 1980, J. Inf. Diseases 141: 362-65; Klimov, et al., 1991, Virus Res. 19: 105-14. In addition, mutations in the M and NP gene could also attenuate influenza virus. Thus, modifications in the M and/or NP genes of influenza may result in generating recombinant influenza viruses useful in inactivated as well as live-attenuated influenza virus vaccines.

In Section 9.2.3 two types of mutagenesis of the M gene are disclosed. In the first, random mutagenesis, a large number of M mutants are generated and introduced back into influenza virus. Interesting candidates with enhanced or reduced replication capacity are selected for vaccine use. In the second, specific mutations are introduced into the M gene to enhance replication or attenuate the virus. For example, a mutant was generated in which base position 15, cytidine, was substituted with uradine to enhance virus replication. Different M gene mutagenesis strategies can be undertaken for the generation of live attenuated influenza virus vaccines. For example, the non-coding sequence of the M gene can be replaced with the influenza B virus non-structural gene's promoter, a strategy known to attenuate influenza virus. Muster, et al., 1991, Proc. Natl. Acad. Sci. 88: 5177-81. Similar strategies can be applied to NP mutagenesis. See Section 5.1.6 for a description of some exemplary strategies.

9.1. NP GENE RESCUE USING ELECTROPORATION

To determine if the rescue process could be carried out using electroporation rather than DEAE-dextran to effect the transfection of RNP complex into helper virus infected cells, the system was initially tested by comparing the electroporation transfection efficiency of NA RNP with that of the DEAE-dextran transfection efficiency of NA RNP. The DEAE-dextran method was used to transfect NA RNP in Section 8.2 above. The results are set forth below in 9.1.1.

9.1.1. ELECTROPORATION OF INFLUENZA VIRUS NA RNP

The rescue of the WSN virus NA gene is based on using a host-range mutant as helper. The influenza virus WSN/HK (see Schulman, et al., 1977, J. Virol. 24: 170-76) derives its NA gene from influenza virus A/HK/68 and its remaining genes from influenza virus WSN. WSN/HK replicates in MDBK cells only in the presence of exogenous protease. Transfectant WSN virus, carrying the WSN NA gene, will replicate in MDBK cells without the addition of exogenous protease. Therefore, MDBK cells in a 6 cm dish were infected with WSN/HK virus at a moi of 1. One hour post infection, the cells were trypsinized, washed with stock solutions of MEM (JRH BioSciences) and PBS (JRH BioSciences), and finally resuspended into a stock solution of PBS at a concentration of 0.1 x 10^7/ml. 0.3 mls of the cell suspension was transferred into 0.4 cm cuvettes (Biorad Laboratories, Richmond, CA).

The RNP complex was reconstituted as follows. The plasmid pT3NAv was constructed as described in Section 8.1.2 and digested with Ksp632I. 0.5 μg of Ksp632I-digested plasmid DNA, 10 μl of purified influenza virus nucleoprotein (NP) and the PA, PB1 and PB2 polymerase proteins (2 μg of total protein), and 2 μl of T3 RNA polymerase (50 U/μl, Stratagene, San Diego, CA) were incubated at 37°C for 15 minutes in the presence of 0.5 mM each of the four nucleoside triphosphates, specifically ATP, GTP, CTP and UTP.

The RNP complex was then transferred to the cuvettes containing the WSN/HK infected MDBK cells in the PBS buffer and the cuvettes were placed in the chamber of a Biorad Gene Pulse Transfection Apparatus (Gene Pulser® model no. 1652076). One pulse of 250 volts, 500 μF (micro Farads), was applied using a BioRad Capacitor Extender (model no. 1652087).

After the electroporation, the cells were plated into new dishes and 2.5 ml of MEM was added to the medium. The transfected cells were incubated at 37°C for 18 hours. The culture medium was harvested and then used in a standard plaque assay in MDBK cells, in accordance with the method disclosed in Tobita, et al. 1975, Med. Microbiol. Immunol. 162: 9-14. The number of infectious viruses was determined 72 hours postinfection after staining with 0.1% crystal violet solution. The results are shown below.

To compare the efficiency of electroporation with that of DEAE-dextran transfection, duplicate samples were subjected to DEAE-dextran transfection following the transfection protocol described in Section 7.1.5.

TRANSFECTION	TITER OF TRANSFECTANT VIRUS	TITER OF HELPER VIRUS	TRANSFECTANT: HELPER RATIO
DEAE-dextran	7.2×10^3	7.5×10^7	1×10^{-4}
Electroporation	1.2×10^4	2×10^6	6×10^{-3}

These results show that in a neuraminidase (NA) RNP rescue system, the total number of transfectants obtained after the electroporation is comparable to that obtained after DEAE-dextran transfection method described in Section 8. However, when electroporation is used to effect transfection, the helper virus replication level is lower than in DEAE-dextran transfection system. Consequently, the ratio of transfectant virus to helper virus in the electroporation system is approximately 50-fold higher than in a DEAE-dextran transfection system. Thus, according to these results, electroporation is a more efficient method for delivering influenza virus RNP inside cells than DEAE-dextran transfection.

Thus, these results demonstrate that RNPs can be transfected into cells by applying a high-voltage pulse to a suspension of the cells and the RNPs. Pulse generators capable of discharging either exponential decay or square wave pulses can be used. Exponential decay pulse generators, such as the Gene Pulser® manufactured by BioRad Laboratories, are preferred.

Although the electroporation as described above was carried out at an actual peak voltage level of 250 volts and a capacitor size of 500 μF, the actual peak voltage used can range from about 150 to about 400 volts in a 0.4 cm cuvette. Additionally, similar results were obtained using a voltage range from about 1000 to about 2000 volts and a capacitor size of 25 μF in a 0.2 cm cuvette. Therefore, voltages in the range of 150 to 2000 volts will work, with capacitance and cuvette size adjusted accordingly as is within the level of skill in the art. In all electroporation experiments

disclosed herein the electroporation was carried at a neutral pH, i.e., 7.2, and room temperature. While temperature is not a critical parameter, the pH of the buffered MDBK cells and RNP complex should be in the neutral range, about 6.8 to about 7.5.

The type of electroporation medium used is an important parameter. Applicants have found that PBS (phosphate buffered saline containing no Ca^{+2} or Mg^{+2}) is a preferred buffer for the electroporation. Stock solutions were used in the disclosed experiments. Lower ionic strength solutions will work; however, with lower ionic strength solutions, such as for example HEPES or phosphate buffered sucrose, resistance increases. Consequently, the relationship between ionic strength and resistance must be taken into account in choosing the buffer. The knowledge required to make such choices is within the level of skill in the art. While PBS is preferred, other media such as HEPES or phosphate buffered sucrose can be used as long as such factors as capacitance and voltage are varied to produce a pulse having a time constant in the same range as the range used with the PBS buffer, i.e., from about 0.5 msec to about 8.2 msec when voltage in the range of about 1000 to about 2000 volts at 25 μF capacitance is applied, or up to about 14 msec when voltage in the range of about 150 to about 400 volts at 500 μF capacitance is applied. Such variations are within the level of skill in the art.

The time period between adding RNP complex to the cuvettes containing the helper infected MDBK cells and pulsing, and the time period between pulsing and plating the transfected cells into new dishes with growth medium should be minimal since delay may cause a drop in transfection efficiency. Periods of up to 5 minutes are acceptable.

9.1.2. PREPARATION OF VIRAL RNA AND CLONING OF NP GENE

The nucleotide sequence of the influenza WSN NP gene is known. Li, et al., 1989, Virus Res. 12:97-112. Influenza WSN viral RNA was prepared and the NP gene was cloned into pUC19 vector as follows. Ten day old embryonated chicken eggs were infected with 100 pfu (plaque forming units) of influenza WSN virus following the method of Burnet, 1936, Br. J. Pathol. 17:282-93. Forty-eight hours later, the allantoic fluid of the infected eggs was harvested and the allantoic fluid was subjected

to centrifugation in a G2-21 centrifuge with a GA-14 rotor at 10,000 rpm for 10 minutes to remove debris. The supernatant was then subjected to a second centrifugation using a L8-70 ultracentrifuge with a SW-28 rotor, at 25K, for 90 minutes. The pellet from the second centrifugation was collected and resuspended in 0.3 ml TMK solution (10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$). 9 μl of 10% SDS and 15 μl of proteinase K (10 mg/ml) was added into the virus suspension, which was then incubated at 56°C for 10 minutes. The influenza viral RNA was extracted with phenol-chloroform.

The viral RNA was then subjected to RT-PCR to amplify the NP sequence. For the reverse transcription, 1 μg of the viral RNA was mixed with 0.1 μg each of oligonucleotides NP1 and NP3 in a total volume of 9.4 μl, and incubated at 84°C for 3 minutes. The reverse transcription and polymerase chain reaction was carried out using RT-PCR amplification kit from Perkin-Elmer Corp., Norwalk, CT, according to the manufacturer's suggested protocol. The primers used in the reverse transcription were:

NP1: 5'GCGCGAATTCTCTTCGAGCAAAAGCAGGGTAGAT3'

NP3: 5'AACTGGAGGACCTATATAC3'.

The primer pairs used in the PCR were:

NP1: 5'GCGCGAATTCTCTTCGAGCAAAAGCAGGGTAGAT3' and

NP2: 5'GTATATAGGTCCTCCAGTTTTCTTAGGATCTTTCCCCGCACT GGGATGTTCTTCCAGATATTT3';

NP3: 5'AACTGGAGGACCTATATAC3' and

NP4: 5'GCGCTCTAGATATTAACCCTCACTAAAAGTAGAAACAAGGG TATT3'.

Primer NP1 contains EcoR1 and Ksp632I restriction enzyme sites, and 18 nucleotides complementary to the 3' end of NP gene. Primer NP2 is complementary to the cRNA of the NP gene from position 273 to 336, except at position 282, in which the adenosine was changed to guanosine. Primer NP2 also contains a EcoO109I site. Thus, the PCR product obtained using primers NP1 and NP2 corresponds to the first 336 nucleotides of NP gene flanked by EcoR1 and EcoO109I sites.

Primer NP3 contains 19 nucleotides identical to cRNA from position 318 to 336 flanked by an EcoO109I site. Primer NP4 contains an XbaI site, T3 RNA polymerase

promoter and 18 nucleotides complementary to 3′ end of cRNA. Thus, the PCR product generated using the primers NP3 and NP4 corresponds to the last 1247 nucleotides (i.e., from the 5′ end) of the NP gene, flanked by EcoO109I and XbaI sites.

The product from the PCR using the primer pair NP1 and NP2 was then digested with EcoRI and EcoO109I, and the product from the PCR using the primer pair NP3 and NP4 was digested with EcoO109I and XbaI. The digested products were then ligated in a trimolecular ligation reaction into EcoRI and XbaI digested pUC19. The resulting plasmid contained the complete nucleotide sequence of the WSN virus NP gene flanked by a T3 RNA polymerase promoter. It also contained a silent mutation engineered into the sequence, replacing the adenosine at position 282 with guanosine. This modification eliminated the XbaI site that was originally present in the cDNA copy of the WSN virus NP gene. The resulting plasmid was called pT3WSN-NPmt.

9.1.3. RESCUE OF TRANSFECTANT VIRUS CARRYING cDNA-DERIVED NP GENE

A temperature sensitive influenza virus, ts56, which has defect in its NP gene, was used to rescue the pT3WSN-NPmt-derived NP gene. The rescue was carried out essentially as described in 9.1.1. MDBK cells in a 6 cm dish were infected with influenza virus ts56 at a moi of 1. One hour post infection, the cells were trypsinized, washed with stock solutions of MEM and PBS and resuspended into 0.3 ml PBS. Cells were transferred into a 0.4 cm cuvette (BioRad Laboratories, Richmond, CA)

The RNP complex was reconstituted as follows. The plasmid pT3NAv was constructed as described in Section 8.1.2 and digested with Ksp632I. 0.5 μg of Ksp632I-digested plasmid DNA, 10 μl of purified influenza virus nucleoprotein (NP) and the three polymerase proteins (2 μg of total protein), and 2 μl of T3 RNA polymerase (50 U/μl, Stratagene, San Diego, CA) were incubated at 37°C for 15 minutes in the presence of 0.5 mM each of the four nucleoside triphosphates, specifically ATP, GTP, CTP and UTP.

The RNP complex was then added to the cuvettes containing the MDBK cells and the cuvettes were placed in the chamber of a Biorad Gene Pulser (model no.

1652076). One pulse of 250 volts, 500 μF, was applied using a BioRad Capacitor Extender (model no. 652087). The cells were then plated into new dishes and 2.5 ml MEM was added. The transfected cells were incubated at 34°C for 18 hours. The culture medium was then used in a standard plaque assay in MDBK cells according to the protocol set forth in Tobita, supra, except that the assay was carried out at 39° C.

Seventy two hours post-infection, visible plaques were isolated with a pipette and were propagated in MDBK cell monolayers as described in Section 8.2.2. Viral RNA was isolated from the propagated viruses as described in Section 9.1.2 and used as a template for RT-PCR. The primers used in the PCR were NP1 and NP4. The PCR products were analyzed by restriction enzyme digestion with XbaI. The XbaI minus products were cloned into pCRII vector (Invitrogen, San Diego, CA), and the nucleotide sequence was confirmed as correct using standard DNA sequencing methods and a commercially available kit from U.S. Biochemicals. As shown below, 5 out of the 36 viruses isolated from the electroporation were transfectants, whereas all 36 viruses derived from DEAE-dextran transfection of duplicate samples, see Section 9.1.1., were helper virus.

TRANSFECTION	VIRUS ANALYZED	TRANSFECTANTS	HELPER
DEAE-dextran	36	0	36
Electroporation	36	5	31

9.2. M GENE RESCUE USING ELECTROPORATION

In this experiment, the M genes of influenza from both the WSN and the PR8 strains were modified to introduce two silent mutations into their respective sequences and rescued using an influenza ts51 virus as helper. The nucleotide sequences of the WSN M gene and the PR8 M gene are known, see Markushin, et al., 1988, Virus Res. 10:263-272 and Winter, et al., 1980, Nuc. Acids Res. 8:1965-74.

9.2.1. PREPARATION OF VIRAL RNA AND CLONING OF THE M GENE

Influenza WSN virus was prepared and the M gene was cloned into pUC19 as follows. Ten day old embryonated chicken eggs were infected with 100 pfu of WSN virus, following the method of Burnet, supra. Forty-eight hours later, the allantoic fluid of the infected eggs was harvested and the allantoic fluid was subjected to centrifugation in a G2-21 centrifuge with a GA-14 rotor at 10,000 rpm for 10 minutes to remove debris. The supernatant was then subjected to a second centrifugation using a L8-70 ultracentrifuge with a SW-28 rotor, at 25K, for 90 minutes. The pellet from the second centrifugation was collected and resuspended in 0.3 ml TMK solution (10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$). 9 μl of 10% SDS and 15 μl of proteinase K (10 mg/ml) was added into the virus suspension, which was then incubated at 56°C for 10 minutes. The influenza viral RNA was extracted with phenol-chloroform.

The viral RNA was then subjected to RT-PCR for amplification of the M sequence, segment 7 of the influenza virus. For the reverse transcription, 1 μg RNA was mixed with 0.1 μg each of oligonucleotides M1 and M3 in a total volume of 9.4 μl, and incubated at 84°C for 3 minutes. The reverse transcription and polymerase chain reaction was carried out using RT-PCR amplification kit from Perkin-Elmer Corp., Norwalk, CT, according to the manufacturer's suggested protocol.

The primers used in the reverse transcription were:

M1: 5'GCGCGAATTCTCTTCGAGCAAAAGCAGGTAGAT3' and

M3: 5'GCGCGGATCCAAATAACATG3'.

The primer pairs used in the PCR were:

M1: 5'GCGCGAATTCTCTTCGAGCAAAAGCAGGTAGAT3' and

M2: 5'GCGCGGATCCCCATTCCCATT3';

M3: 5'GCGCGGATCCAAATAACATG3' and

M4: 5'GCGCTCTAGATATTAACCCTCACTAAAAGTAGAAACAAGGT AGTT3'.

Primer M1 contains EcoRI and Ksp632I sites, and 17 nucleotides complementary to 3' end of M gene. Primer M2 is complementary to cRNA of M gene at positions 278-294 except positions 286 and 289, in which two silent

mutations were introduced (C286-T, A289-G). A BamHI site was also included in primer M2. PCR product generated by using these primers contains the first 294 nucleotides of the M gene flanked by EcoRI and BamHI sites.

Primer M3, which contains a BamHI site, is identical to cRNA at positions 289-304. Primer M4 contains XbaI site, T3 RNA promoter and 18 nucleotides complementary to 3' end of the cRNA of M gene. The PCR product using primers M3 and M4 contains the 737 nucleotides of the 5' end of the M gene flanked by BamHI and XbaI sites.

The product from the PCR using the primer pair M1 and M2 was digested with EcoRI and BamHI. The product from the PCR using the primer pair M3 and M4 was digested with BamHI and XbaI restriction enzymes. These products were then ligated in a trimolecular-reaction into EcoRI and XbaI digested pUC19. The resulting plasmid, pT3WSN-Mmt, contained the complete nucleotide sequence of the M gene of the influenza WSN virus, a truncated T3 bacteriophage RNA polymerase promoter and a Ksp632I restriction enzyme site. In addition, it contained two introduced silent mutations at positions 286 (C-T) and 289 (A-G). The latter mutation created the unique BamHI site at position 289.

The same process was used to generate plasmid pT3PR8-Mmt, containing the complete nucleotide sequence of the M gene from influenza PR8 virus also containing the identical silent mutations, the truncated T3 promoter and the Ksp632I site, using influenza PR8 virus as the starting material.

9.2.2. RESCUE OF TRANSFECTANT VIRUS CARRYING cDNA DERIVED M GENE

The helper virus used in this system is a temperature sensitive mutant ts51, which has defect on the M gene. See Rey, et al., 1992, J. Virol. 66: 5815-24. MDBK cells in a 6 cm dish were infected with influenza virus ts51 at a moi of 1. One hour post infection, the cells were trypsinized, washed with stock solutions of MEM and PBS and resuspended into 0.3 ml PBS. Cells were transferred into 0.4 cm cuvettes (BioRad Laboratories, Richmond, CA) RNP complex was reconstituted using Ksp632I-digested plasmid DNA from plasmids pT3PR8-Mmt and pT3WSN-Mmt and the rescue of M genes from both plasmids was effected and the results analyzed according to the procedure described in Section 9.1.3. Viral RNA from the WSN and PR8 transfections

was purified as described in Section 9.1.2 and used as the template for reverse transcription and PCR amplification of the M gene using the primers M1 and M4. The WSN PCR products were then analyzed by restriction enzyme digestion using BamHI and HindIII. Positive digestion using both enzymes suggested that the M gene in the transfected cells was derived from pT3WSN-Mmt. With respect to the PR8 M gene transfectants, the BamHI positive PCR products were cloned into pCRII vector (Invitrogen), and sequenced using the standard protocol for positive confirmation of rescued PR8 M gene sequence. As shown below, the majority of viruses derived from electroporation were transfectants, whereas most of the viruses derived from DEAE-dextran transfection were helper virus.

TRANSFECTION	VIRUS ANALYZED	TRANSFECTANTS	HELPER	VIRUS WITH RNP DERIVED M GENE
DEAE-dextran	36	8	25	3
Electroporation	36	31	2	3

9.2.3. MUTAGENESIS OF M GENE FOR GENERATION OF HIGH-GROWING INFLUENZA VIRUS

In this experiment, site-specific mutagenesis was used to introduce modifications into the native M gene sequence. A plasmid, designated pT3PR8-Mh1, was constructed in accordance with Section 9.2.1, in which the cytidine residue at position 15 was changed to uridine in order to enhance the transcription activity of the influenza virus. Plasmid RNA is then rescued using ts51 as helper virus according to the protocol described in Section 9.2.2.

A second mutant can be constructed in which the M gene is present in the influenza virus in two copies. This is achieved by using the internal ribosome entry site of BIP protein placed between two identical M genes, allowing translation of both copies of the M gene in a bicistronic construct in accordance with the procedures and methods described in Garcia-Sastre, 1994, J. Virol 68:6254-6251. Plasmid RNA is

then rescued using ts51 as helper virus according to the protocol described in Section 9.2.2.

Because avian influenza viruses replicate in embryonated eggs to very high titer, modifications incorporating residues native to the avian influenza sequence would be expected to enhance the replication of the human influenza viruses, thereby increasing yields. For example, the nucleotides encoding one or more amino acid residues of the PR8 strain (or another human strain, for example the WSN strain) of influenza can be replaced with nucleotides encoding an amino acid residue or residues that are present in the corresponding positions in the avian strain of the virus. Exemplary amino acid residues in the M1 protein of the PR8 strain that can be replaced with their avian counterpart residues include those at position 59 (isoleucine replaced with valine), position 115 (isoleucine replaced with valine), 121 (alanine replaced with threonine) and position 137 (alanine replaced with threonine). Exemplary residues in the M2 protein of the PR8 strain that can be replaced with their avian counterpart residues include those at position 11 (isoleucine replaced with threonine), position 14 (glutamic acid replaced with glycine), position 16 (cysteine replaced with glutamic acid) and position 55 (phenylalanine replaced with leucine). Determination of the identity of the nucleotide sequences that will result in such modifications in the encoded amino acid sequences are well within the skill in the art. Mutants are rescued using the techniques described in Example 9.2.2. The replication level of those mutant viruses are characterized in embryonated eggs following the procedures of Barrett et al., Chapter 6, pages 119-150, in Virology: A Practical Approach, ed. Mahy, IRL Press, Washington D.C., 1985 . Mutants replicating at high titers can be used for the manufacture of inactivated or live vaccines.

PCR based random-mutagenesis of the M gene can also be employed to generate high-growing influenza virus. The mutant M sequences created by random mutagenesis can be rescued in accordance with Section 9.1.3 and high-growing mutants selected by serial passage in eggs.

10. DEPOSIT OF MICROORGANISMS

An E. coli cell line containing the plasmid pIVACAT is being deposited with the Agricultural Research Culture Collection (NRRL), Peoria, IL; and has the following accession number

Strain	Plasmid	Ascension Number
E. coli (DH5a)	pIVACAT	NRRL

The present investigation is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any constructs, viruses or enzymes which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for producing a chimeric influenza virus comprising culturing a host cell transfected with a recombinant RNP and infected with a parental strain of influenza and recovering the chimeric influenza virus from the culture, said recombinant RNP having been transfected into said host cell by electroporation.

2. A method of claim 1, said electroporation being carried out at an actual peak voltage in the range of about 150 about 400 volts with electric capacitance of about 500 μF.

3. A method of claim 1, said electroporation being carried out at an actual peak voltage in the range of about 1000 to about 2000 volts with electric capacitance of about
25 μF.

4. A method for gene expression comprising culturing a host cell transfected with a recombinant RNP so that the heterologous gene is expressed in the culture, said recombinant RNP having been transfected into said host cell by electroporation.

5. A method of claim 4, said electroporation being carried out at an actual peak voltage in the range of about 150 to about 400 volts with electric capacitance of about 500μF.

6. A method of claim 4, said electroporation being carried out at an actual peak voltage in the range of about 1000 to about 2000 volts with electric capacitance of about
25 μF.

7. A chimeric virus comprising influenza virus containing a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to an influenza viral polymerase binding site, said heterologous RNA

sequence comprising an RNA sequence coding on expression for an influenza M protein.

8. A chimeric virus of claim 7 wherein said heterologous RNA sequence comprises an RNA sequence coding on expression for an influenza M protein, said sequence containing at least one substitution of a native residue with a non-native residue.

9. A chimeric virus comprising influenza virus containing a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to an influenza viral polymerase binding site, said heterologous RNA sequence comprising an RNA sequence coding on expression for an influenza NP protein.

10. A chimeric virus of claim 9 wherein said heterologous RNA sequence comprises an RNA sequence coding on expression for an influenza NP protein, said sequence containing at least one substitution of a native residue with a non-native residue.

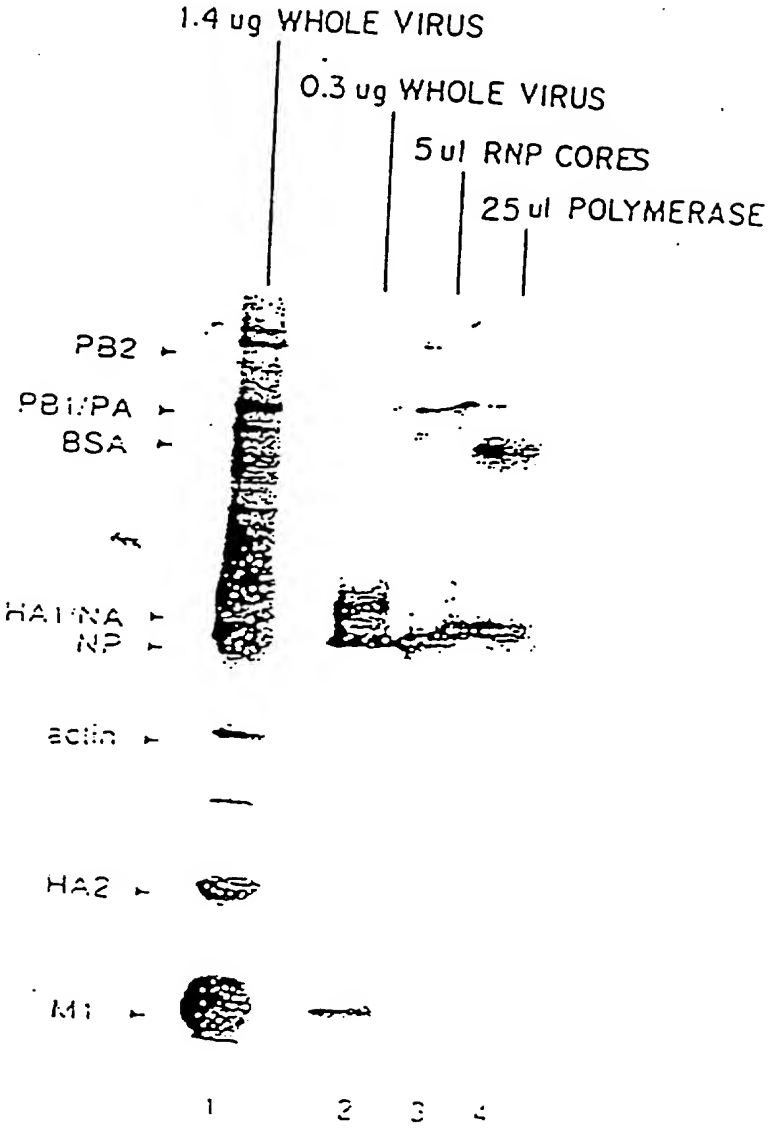

1.4 ug WHOLE VIRUS
0.3 ug WHOLE VIRUS
5 ul RNP CORES
25 ul POLYMERASE
PB2
PB1/PA
BSA
HA1/NA
NP
actin
HA2
M1
1
2
3
4

FIG. 1

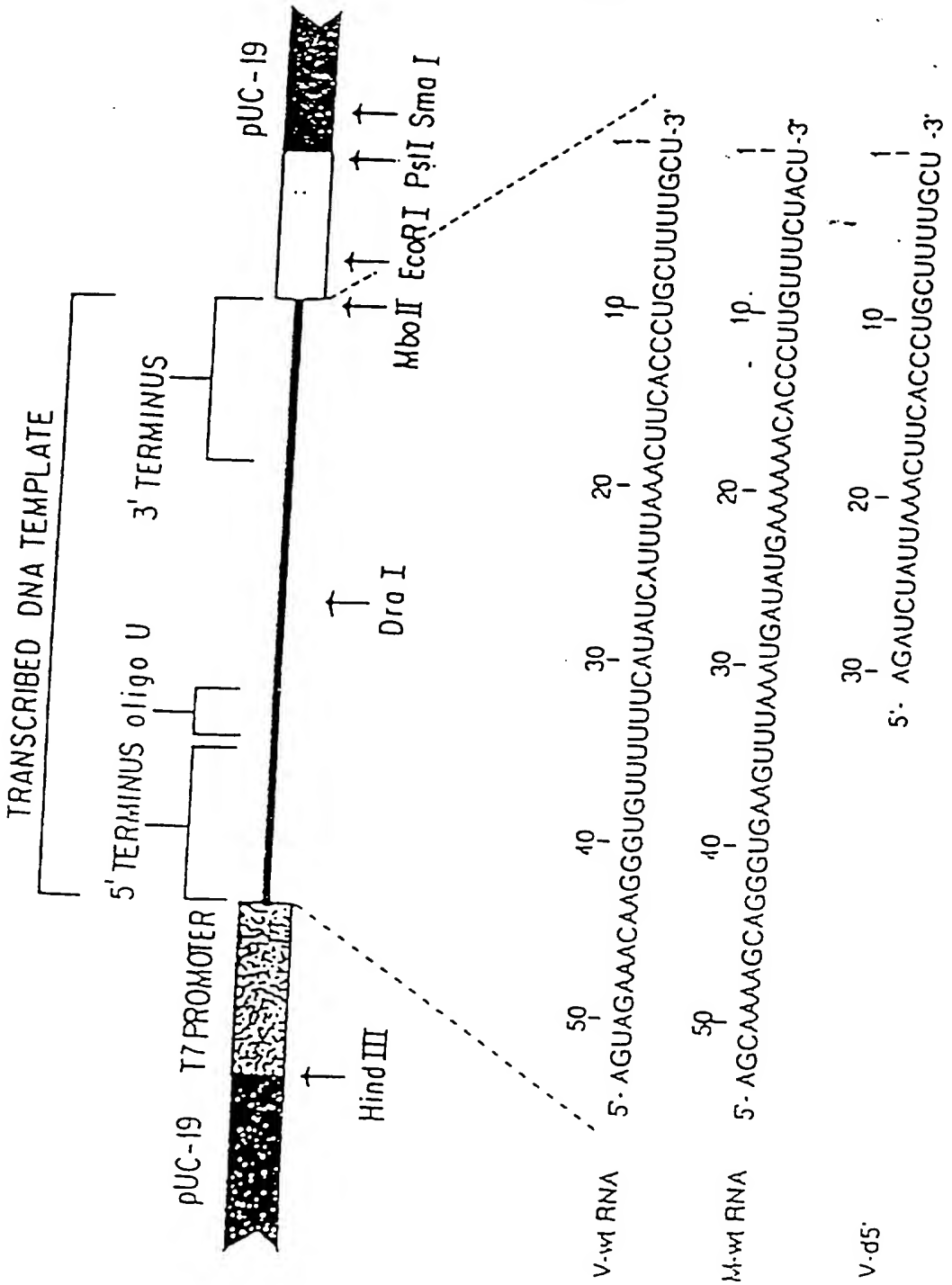

TRANSCRIBED DNA TEMPLATE
5' TERMINUS oligo U
3' TERMINUS
pUC-19
T7 PROMOTER
pUC-19
Hind III
Dra I
MboII EcoRI PstI Sma I
V-wt RNA
5- AGUAGAAACAAGGGUGUUUUUUCAUAUCAUUUAAACUUCACCCUGCUUUUGCU-3'
M-wt RNA
5- AGCAAAAGCAGGGUGAAGUUUAAAUGAUAUGAAAAAACACCCUUGUUUCUACU-3'
V-d5'
5- AGAUCUAUUAAAACUUCACCCUGCUUUUGCU -3'

FIG. 2

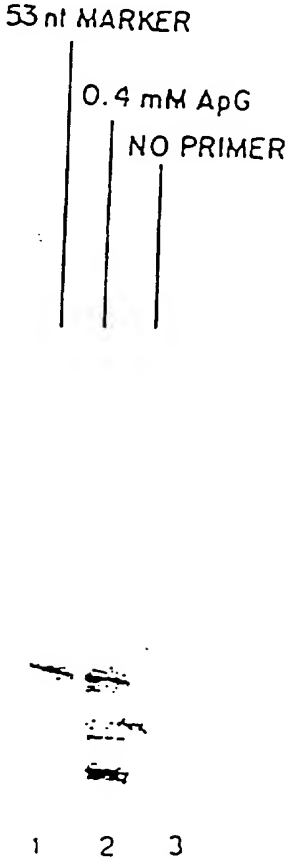


FIG. 3A

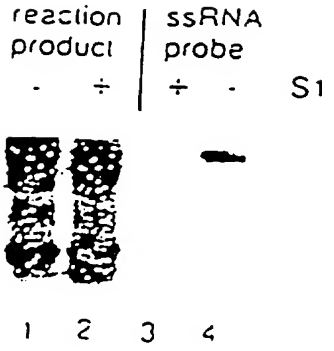


FIG. 3B

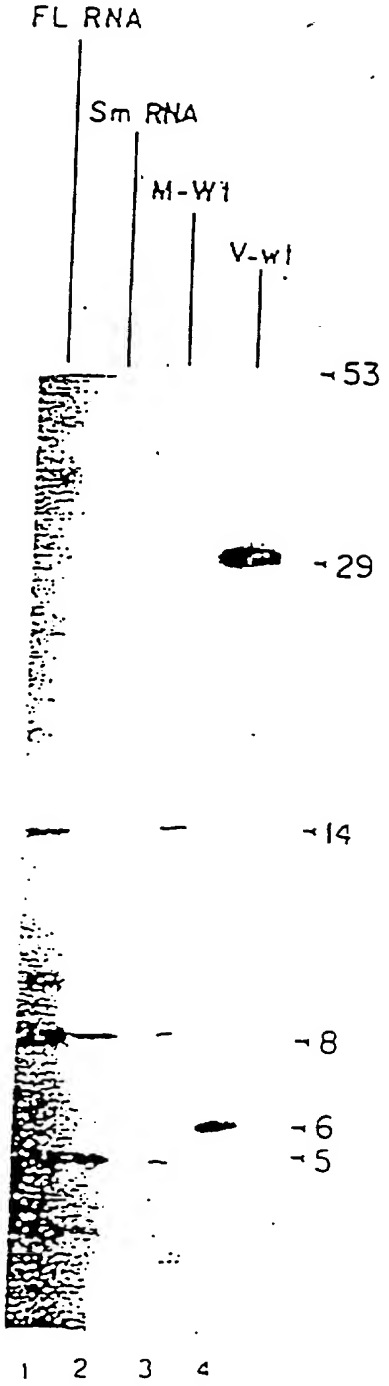


FIG. 3C

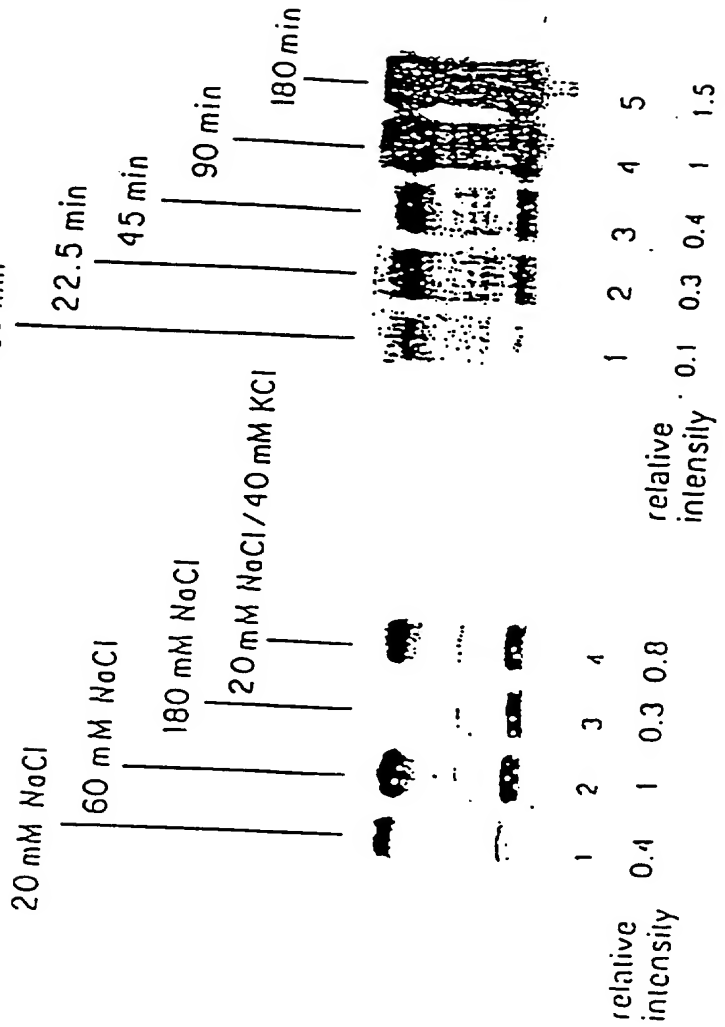


FIG. 4C

FIG. 4B

FIG. 4A

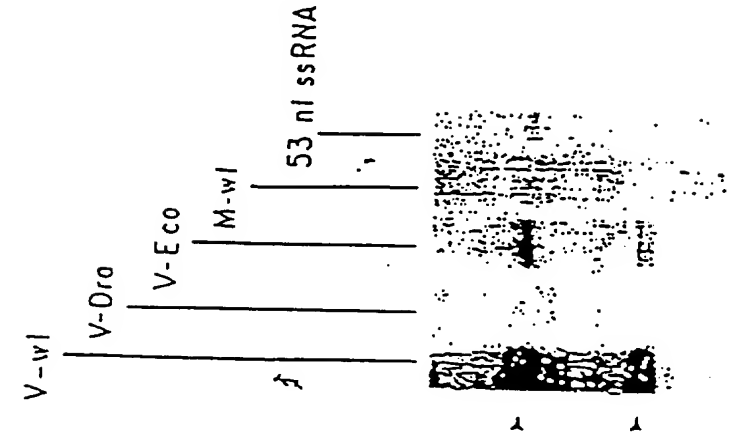

53 nl ssRNA
M-wI
V-Eco
V-Dra
V-wI
1 2 3 4 5

FIG. 5B

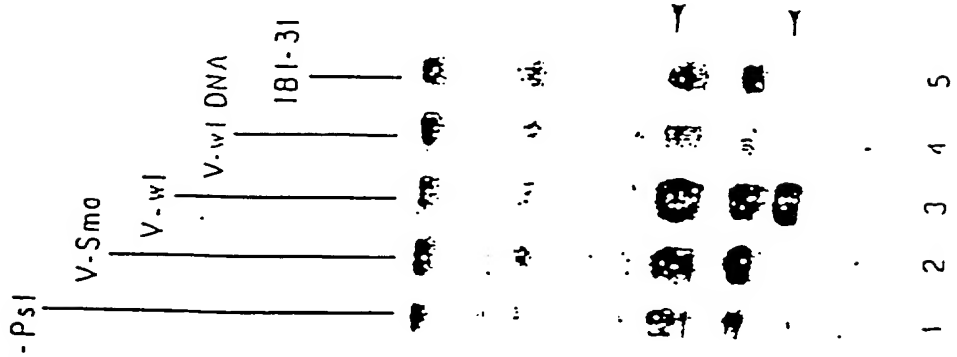
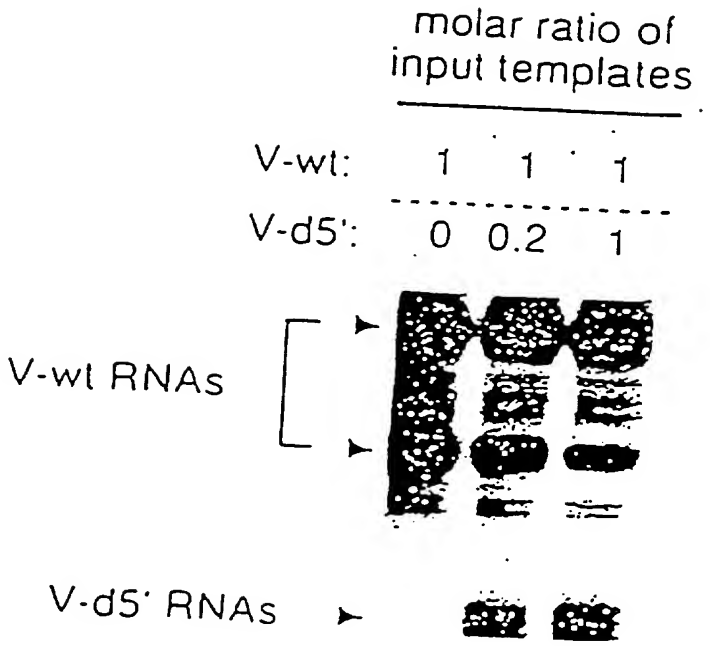

181-31
V-wI DNA
V-wI
V-Smo
V-PsI
1 2 3 4 5

FIG. 5A



corrected relative intensities				
		1	2	3
	V-wt:	1	0.8	0.6
	V-d5':	0	0.2	0.4

FIG. 6

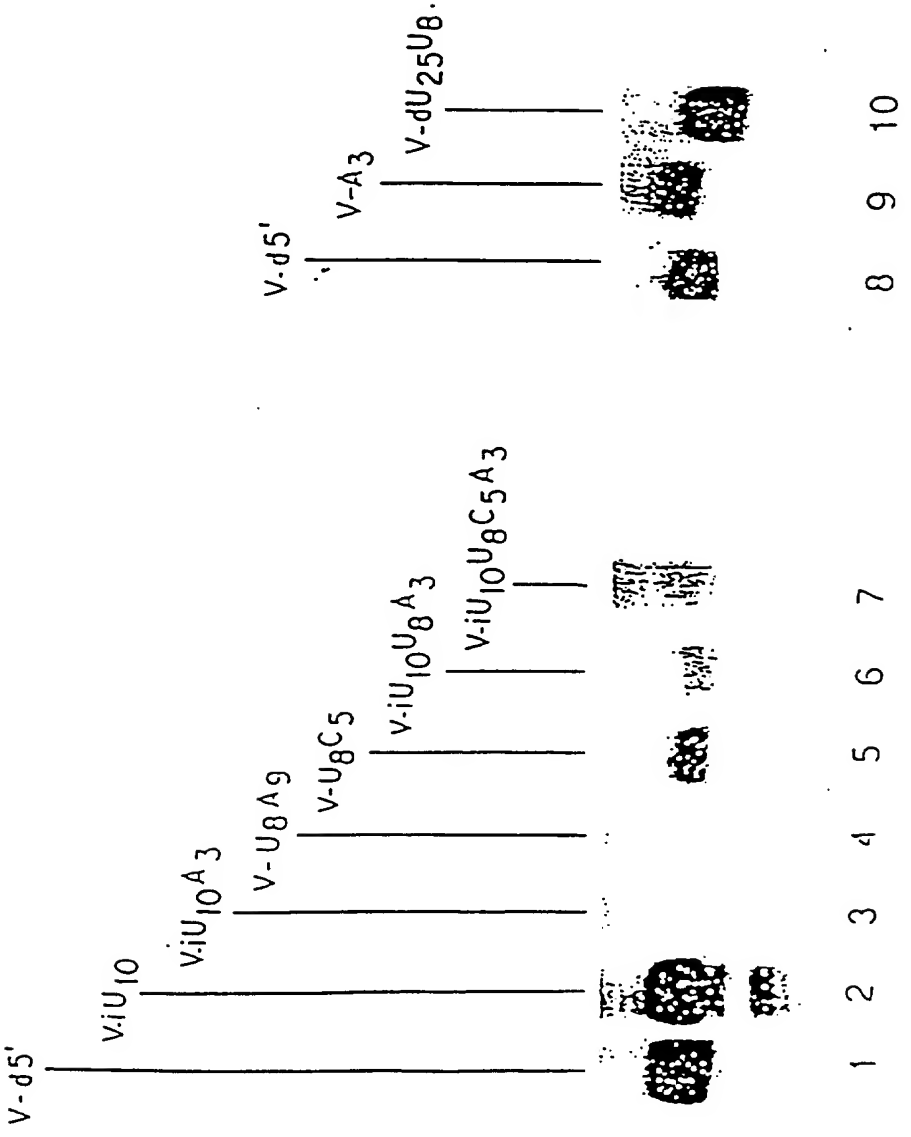

V-d5'
V-iU10
V-iU10A3
V-U8A9
V-U8C5
V-iU10U8A3
V-iU10U8C5A3
V-d5'
V-A3
V-dU25U8.
1 2 3 4 5 6 7 8 9 10

FIG. 7

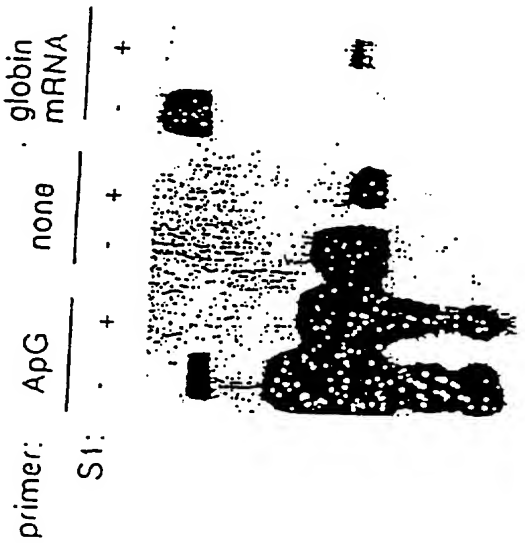

primer:
S1:
ApG
none
globin mRNA
1 2 3 4 5 6

FIG. 8B

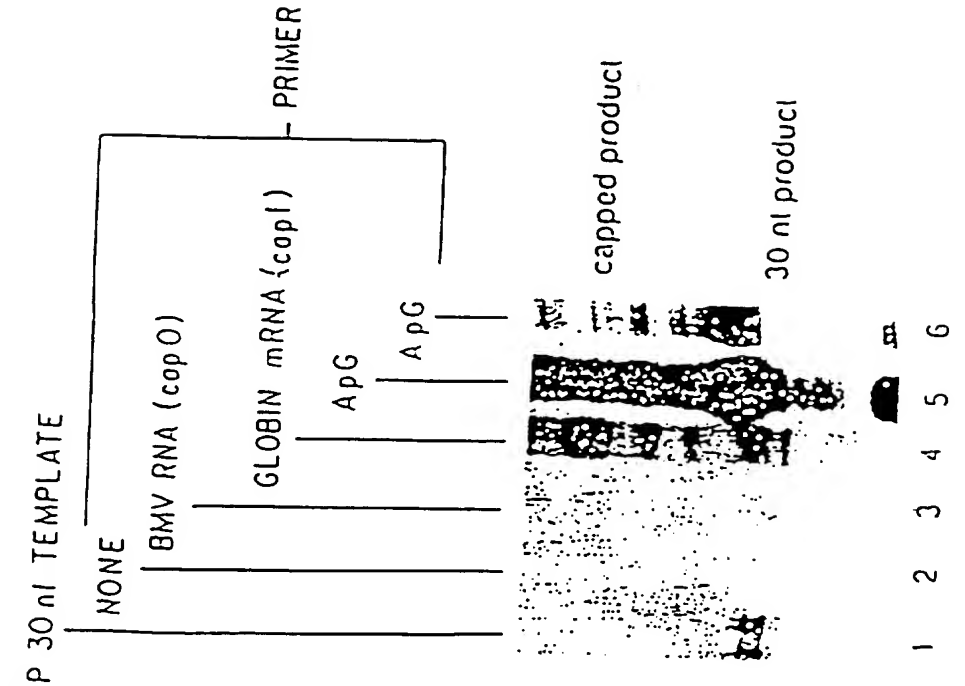

32P 30 nl TEMPLATE
NONE
BMV RNA (cap 0)
GLOBIN mRNA (cap 1)
ApG
ApG
PRIMER
capped product
30 nl product
1 2 3 4 5 6

FIG. 8A

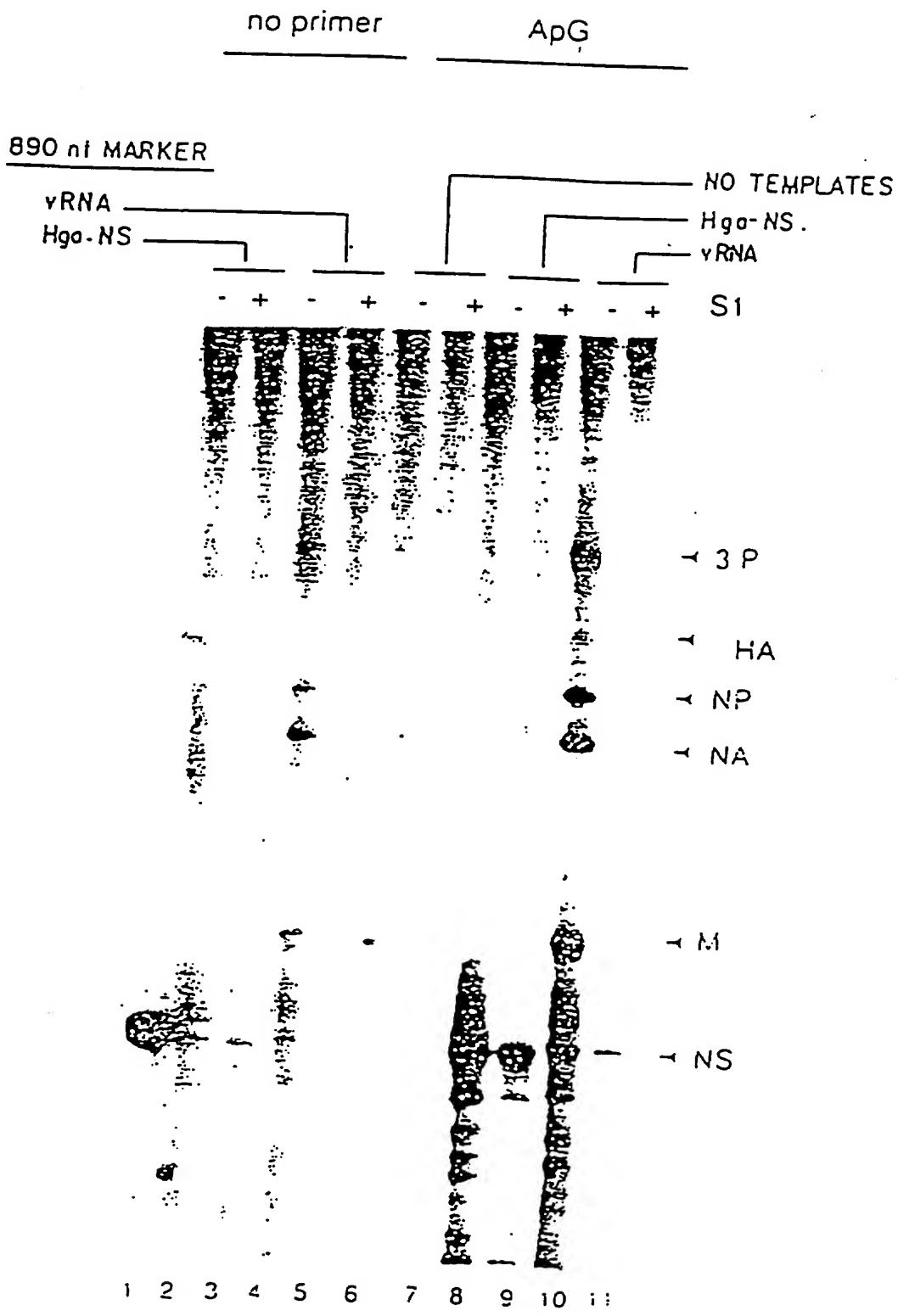
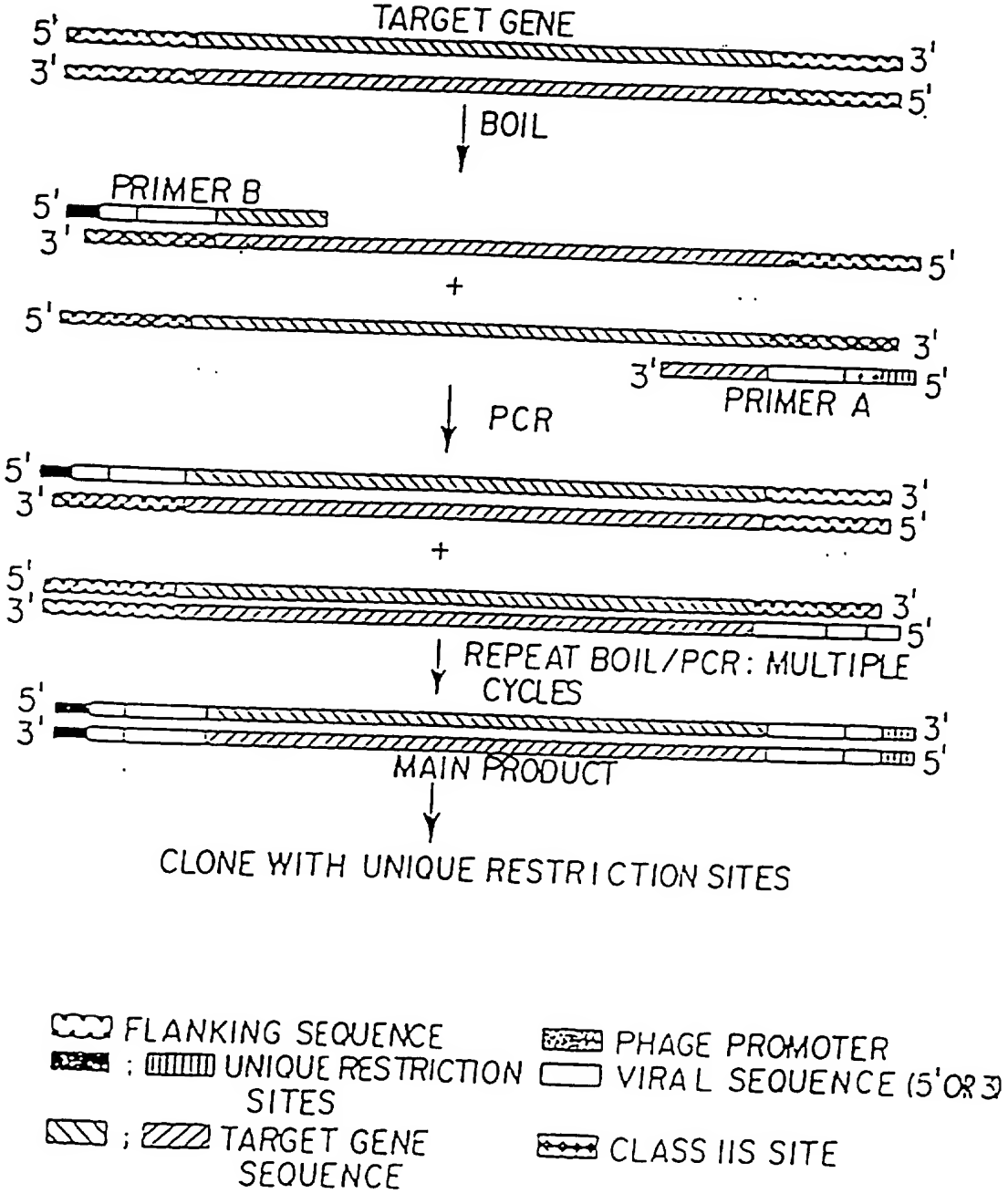

no primer
ApG
890 nt MARKER
NO TEMPLATES
vRNA
Hgo-NS
Hgo-NS.
vRNA
- + - + - + - + - + S1
3 P
HA
NP
NA
M
NS
1 2 3 4 5 6 7 8 9 10 11

FIG. 9

FIG. 10


TARGET GENE
5'
3'
3'
5'
BOIL
PRIMER B
5'
3'
5'
+
5'
3'
3'
5'
PRIMER A
PCR
5'
3'
3'
5'
+
5'
3'
3'
5'
REPEAT BOIL/PCR: MULTIPLE CYCLES
5'
3'
3'
5'
MAIN PRODUCT
CLONE WITH UNIQUE RESTRICTION SITES
FLANKING SEQUENCE
: UNIQUE RESTRICTION SITES
; TARGET GENE SEQUENCE
PHAGE PROMOTER
VIRAL SEQUENCE (5' OR 3')
CLASS IIS SITE

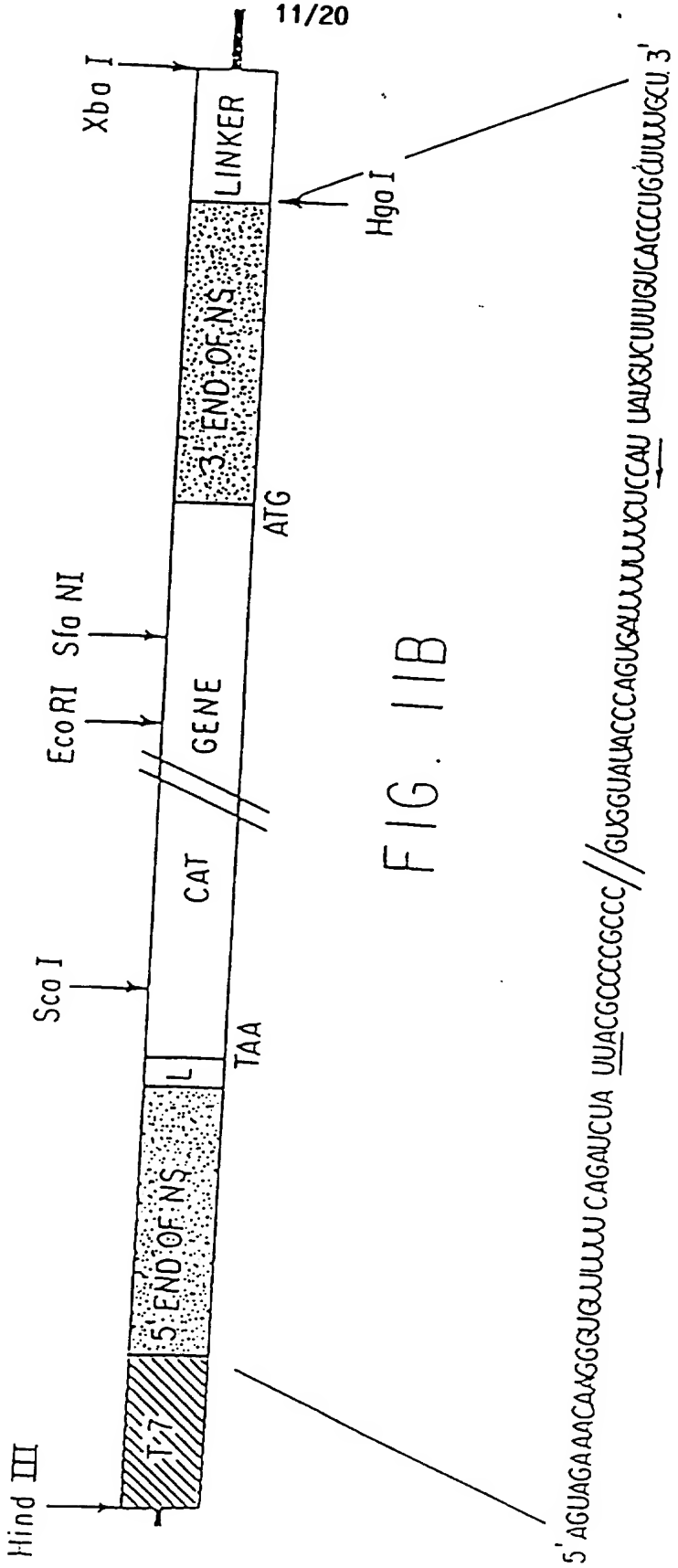


FIG. IIA

FIG. IIB

FIG. 12

1 2 3 4 5 6

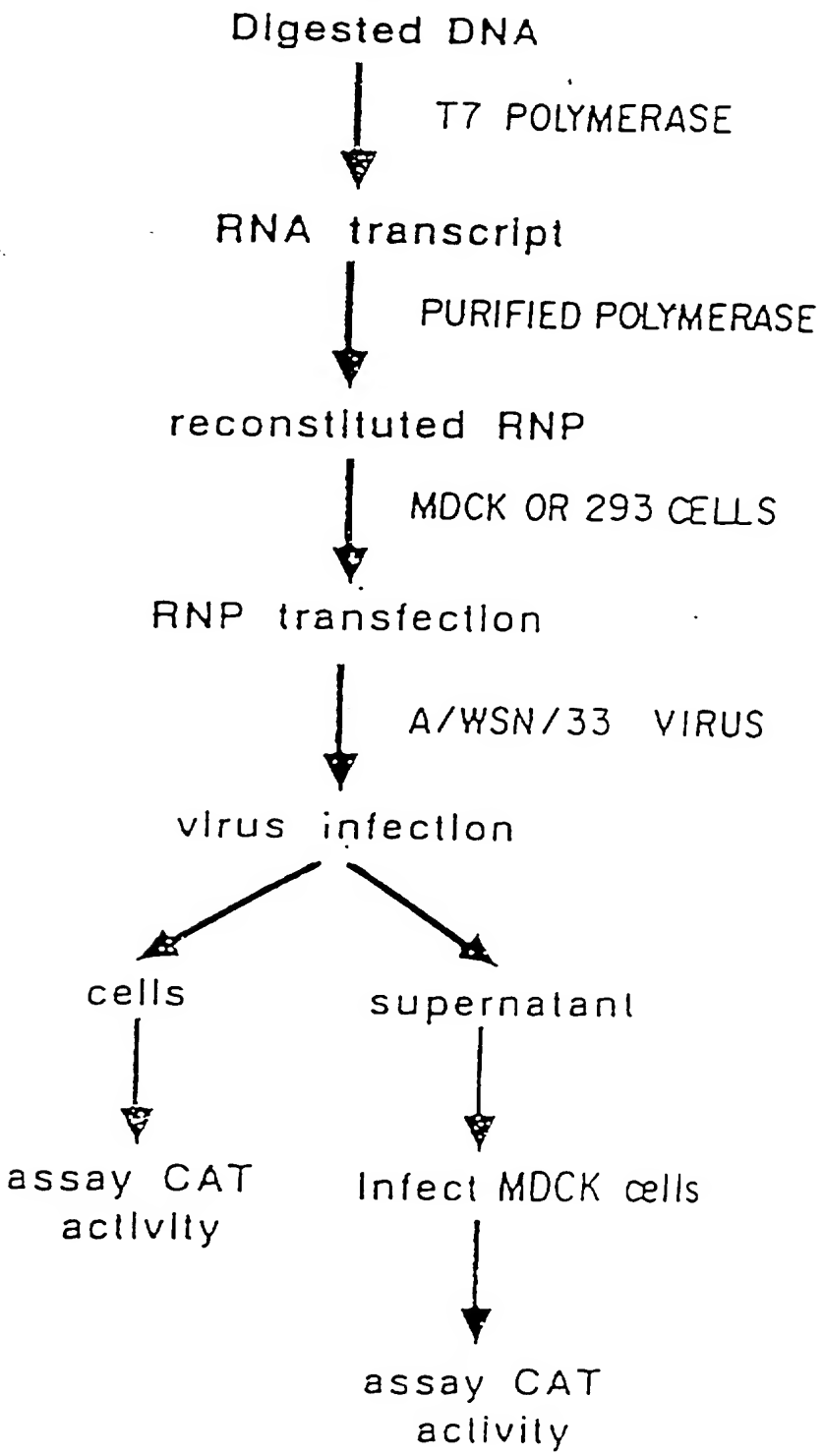
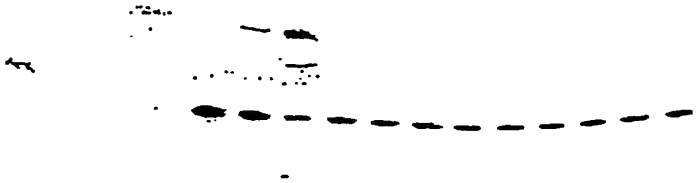

Digested DNA
T7 POLYMERASE
RNA transcript
PURIFIED POLYMERASE
reconstituted RNP
MDCK OR 293 CELLS
RNP transfection
A/WSN/33 VIRUS
virus infection
cells
supernatant
assay CAT activity
Infect MDCK cells
assay CAT activity

FIG. 13

	C	1	2	3	4	5	6	7	8	9	10	11	12	13	14
TIME	-1:5		-1	0	0	1	1	3	3	5	5	7	7	9	9
RNA	-		-	+	-	+	-	+	-	+	-	+	-	+	-
Pol	-		-	+	-	+	-	+	-	+	-	+	-	+	-
WSN	-		-	-	-	+	+	+	+	+	+	+	+	+	+

FIG. 14a



	C	1	2	3	4	5	6	7	8	9	10	11	12
TIME		3	7	7	3	7	3	7	3	7	7	3	7
RNA		+	+	+	+	+	+	+	+	+	-	-	-
Pol		+	+	+	+	+	-	-	-	-	-	-	-
WSN		+	+	+	-	-	+	+	-	-	+	-	-
Pre-inc		+	+	-	+	+	-	-	-	-	-	-	-

FIG. 14b

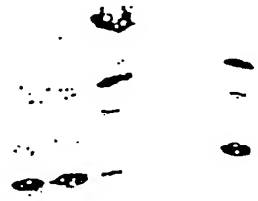
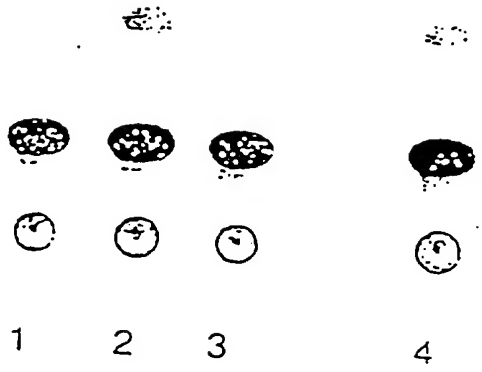
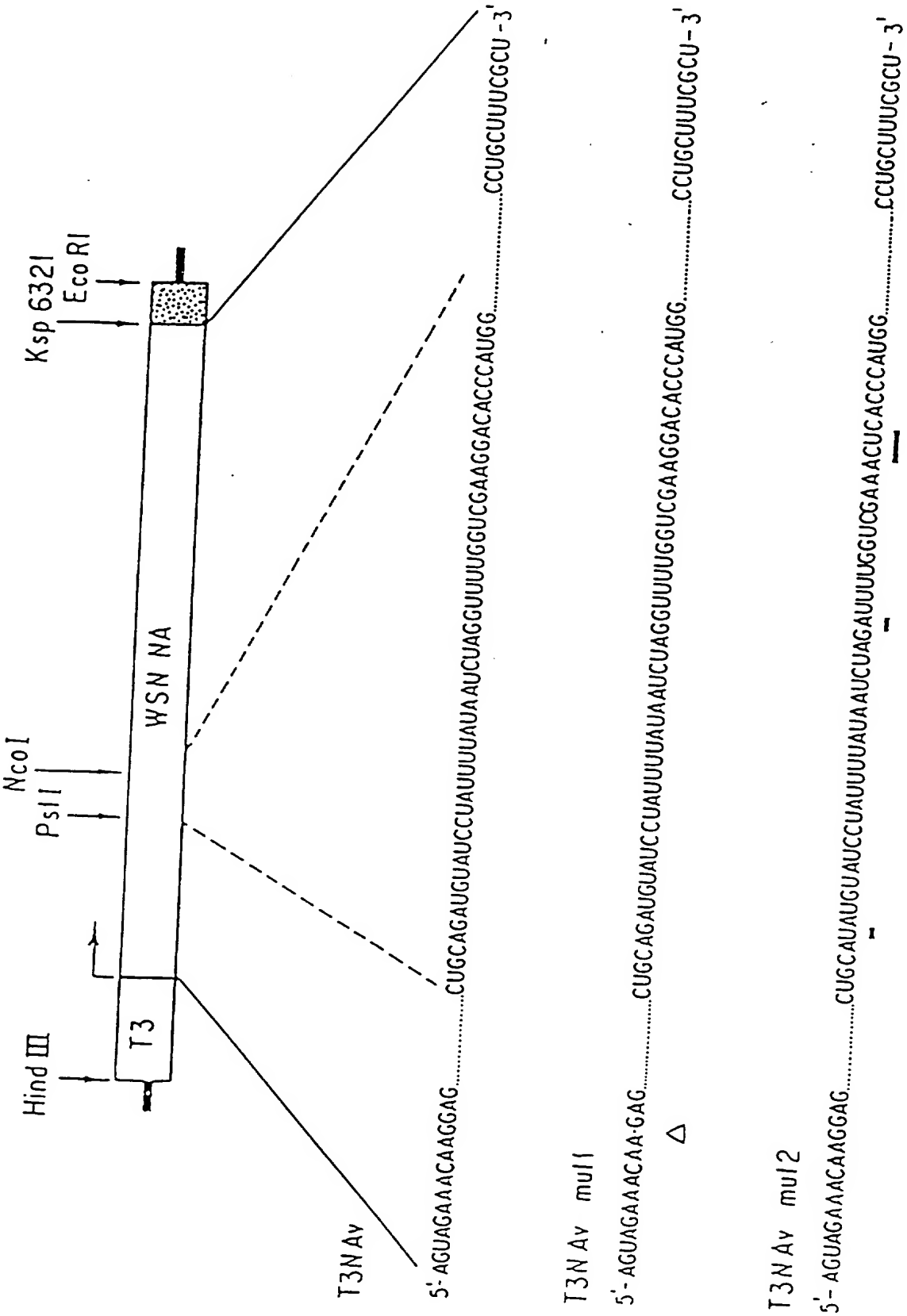


FIG. 14c

	1	2	C	3
	7	3		7
RNP TRANSFECTION	-1	-1		-2

FIG. 15




Hind III
T3
Psi I
Nco I
WSN NA
Ksp 6321
Eco RI
T3N Av
5'-AGUAGAAACAAGGAG.....CUGCAGAUGUAUCCUAUUUUAUAAUCUAGGUUUUGGUCGAAGGACACCCAUGG.....CCUGCUUUCGCU-3'
T3N Av mut1
5'-AGUAGAAACAA-GAG.....CUGCAGAUGUAUCCUAUUUUAUAAUCUAGGUUUUGGUCGAAGGACACCCAUGG.....CCUGCUUUCGCU-3'
Δ
T3N Av mut2
5'-AGUAGAAACAAGGAG.....CUGCAUAUGUAUCCUAUUUUAUAAUCUAGAUUUUGGUCGAAACUCACCCAUGG.....CCUGCUUUCGCU-3'

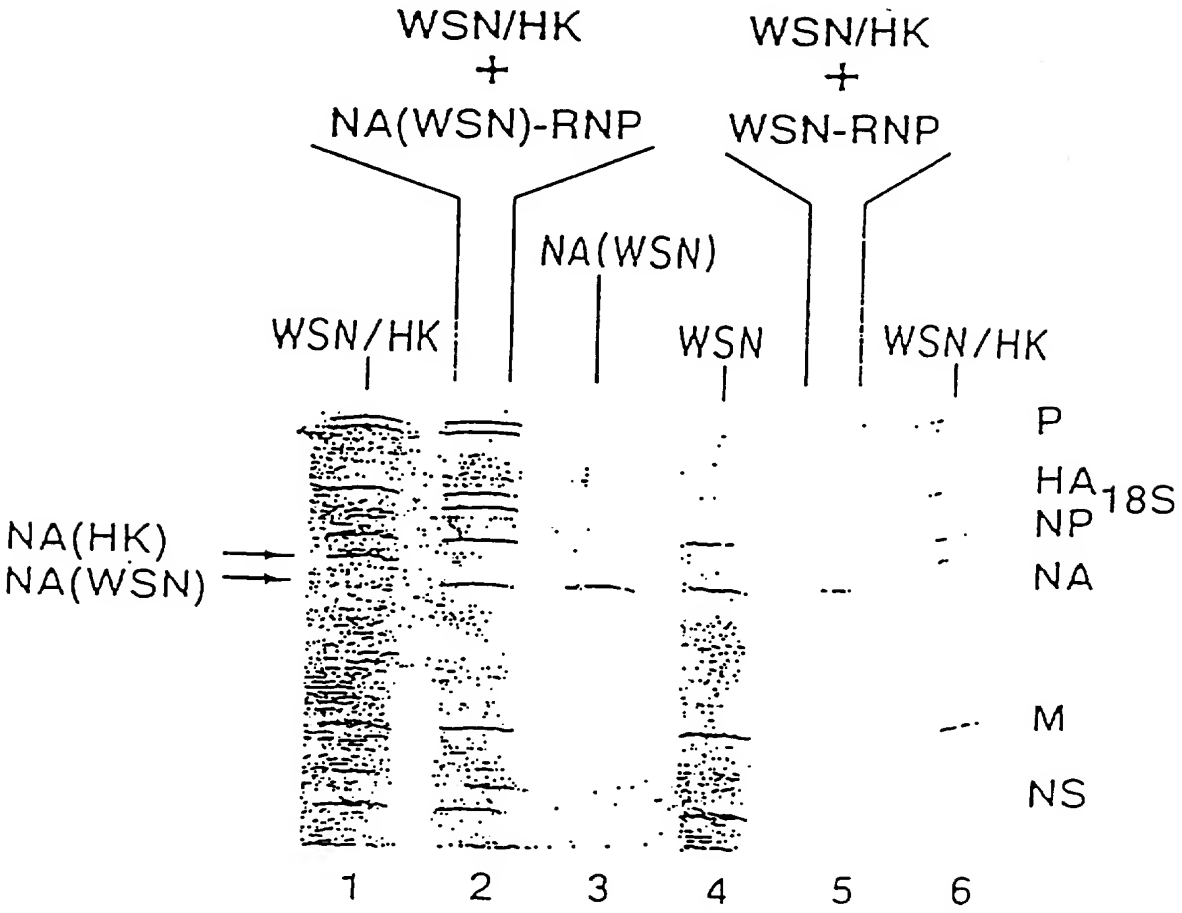

WSN/HK
+
NA(WSN)-RNP
WSN/HK
+
WSN-RNP
NA(WSN)
WSN/HK
WSN
WSN/HK
P
HA
NP
18S
NA
M
NS
NA(HK)
NA(WSN)
1
2
3
4
5
6

FIG. 17

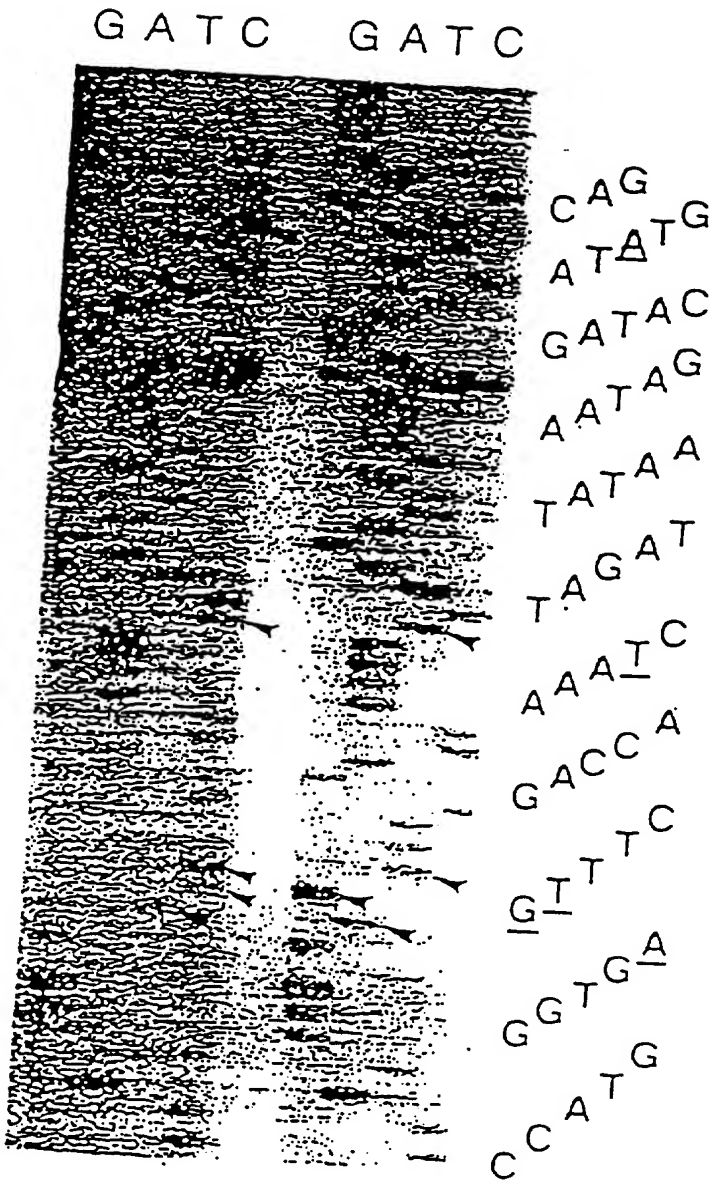
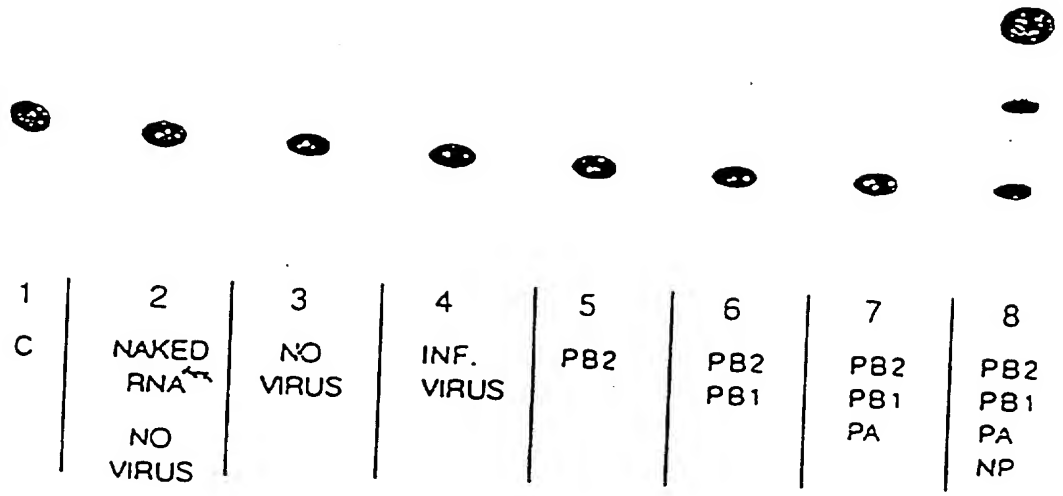

GATC GATC
CAG
ATATG
GATAC
AATAG
TATAA
TAGAT
AAATC
GACCA
GTTTC
GGTGA
CCATG

FIG. 18

FIG. 19


1
C
2
NAKED RNA
NO VIRUS
3
NO VIRUS
4
INF. VIRUS
5
PB2
6
PB2
PB1
7
PB2
PB1
PA
8
PB2
PB1
PA
NP

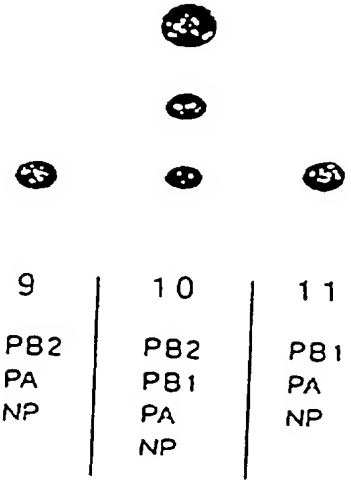

9
PB2
PA
NP
10
PB2
PB1
PA
NP
11
PB1
PA
NP

FIG. 20a

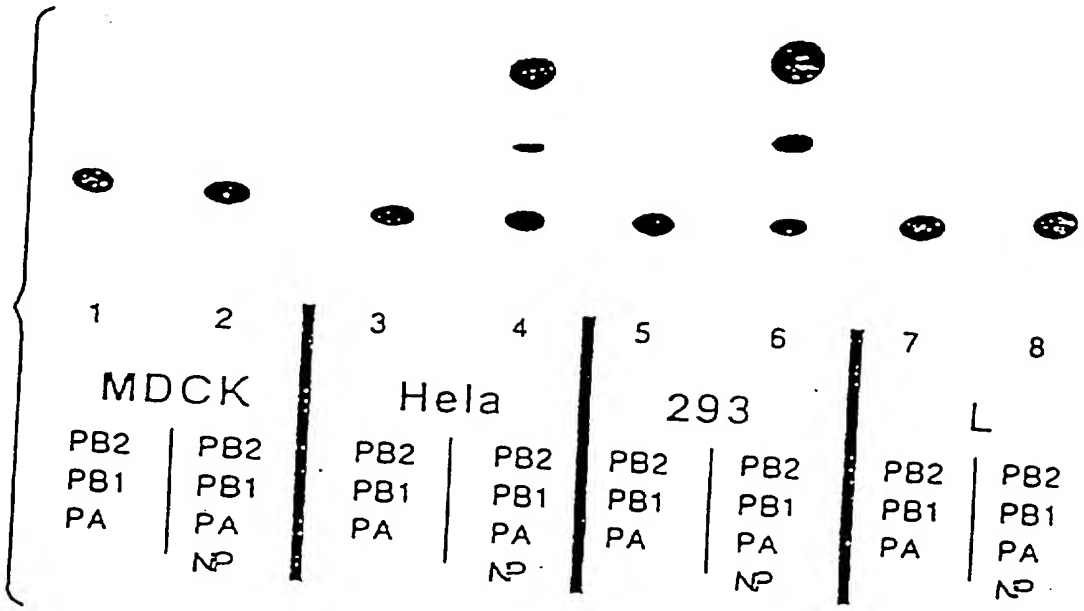
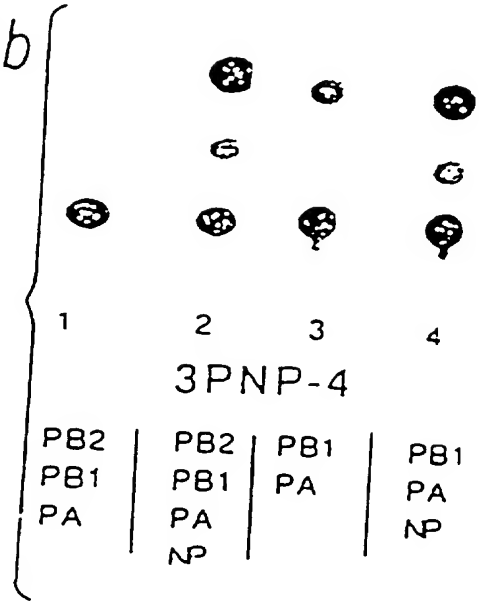


FIG. 20b



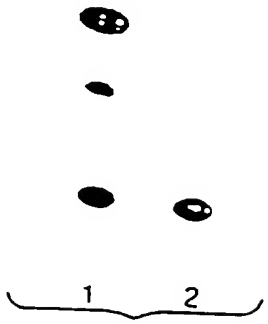


FIG. 20c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12559

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 13/00, 7/01
US CL :435/173.6, 173.3, 172.3, 235.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/173.6, 235.1, 69.1, 91.32, 172.3, 173.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,166,057 (PALESE ET AL), 24 NOVEMBER 1994, see entire document.	1-6
Y	Analytical Biochemistry, volume 174, issued 1988, Potter, "Electroporation in Biology: Methods, Applications, and Instrumentation", see entire document, especially Table 1 and page 369, column 1.	1-6
Y	US, A, 5,306,631 (HARRISON ET AL), 26 APRIL 1994, see column 20, lines 33-34 and column 17, lines 4-29.	1-6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 JANUARY 1996	15 FEB 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MARY E. MOSHER
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12559

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Virology, volume 68, number 1, issued January 1994, Smeek et al, "The Influenza Virus Variant A/FM/1/47-MA Possesses Single Amino Acid Replacements in the Hemagglutinin, Controlling Virulence, and in the Matrix Protein, Controlling Virulence as well as Growth", pages 530-534, see entire document, especially the Abstract and the last four lines of Table 1.	7, 8
X	Virology, volume 181, issued 1991, Mandler et al, "Mutants and Revertants of an Avian Influenza A virus with Temperature-Sensitive Defects in the Nucleoprotein and PB2", see entire document.	9, 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12559

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12559

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, medline. Search terms: influenza, m, matrix, recombinant, chimer?, mutant, mutants, mutated, mutation, np, nucleoprotein#, electroporat?, mammal?, protein#, polypeptide#, complex, dna, rna, nucleic, nucleotide, ribonucleoprotein#, electric#####, electro#####, rnp.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group 1, claim(s)1-6, drawn to a method for producing a chimeric influenza virus comprising electroporation.
Group 2, claim(s) 7 and 8, drawn to chimeric influenza virus comprising heterologous M gene.
Group 3, claim(s) 9 and 10, drawn to chimeric influenza virus comprising heterologous NP gene.
The inventions listed as Groups 1-3 do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The general method of group 1 contains no special technical feature in common with groups 2 and 3. The special technical feature of group 2, a heterologous M gene, is different from the special technical feature of group 3, a heterologous NP gene. Therefore these groups lack the same or corresponding special technical features.